The Development of Resistance to Quinolones in *Staphylococcus aureus*

Esther Jane Durham

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Abstract

The quinolones are a class of antibiotics that target DNA gyrase and topoisomerase IV, enzymes involved in DNA replication. DNA gyrase has two A subunits encoded by *gyrA*, and two B subunits encoded by *gyrB*. Topoisomerase IV is also tetrameric, and is encoded by *grlA* and *grlB* in *Staphylococcus aureus*. Previous research has suggested that DNA gyrase is the primary quinolone target in Gram-negative organisms while topoisomerase IV is the primary target in Gram-positive organisms. In *S. aureus*, resistance to the quinolones arises through mutations within the quinolone resistance determining regions (QRDRs) of the *gyrA* or *grlA* genes. However, low-level resistance has also been attributed to mutations in *gyrB* or *grlB*, and with enhanced expression of cell surface efflux proteins.

Historically, the quinolones have had good activity against Gram-negative organisms but have been less effective against Gram-positive organisms. Moxifloxacin is a new 8-methoxyquinolone that has good activity against Gram-positive organisms. In this thesis the activity of moxifloxacin against sensitive laboratory strains and clinical isolates of *S. aureus* was investigated by time-kill kinetics. The bactericidal activity of moxifloxacin under different growth conditions was established in order to determine the mechanisms of killing action. Subsequently, the killing kinetics against laboratory derived mutants with characterised mutations within the QRDRs of *gyrA* and *grlA* were also investigated. Mutations within *gyrA* and *grlA* did not alter the pattern of killing by moxifloxacin.

To investigate the sequential development of resistance a series of low-, medium- and high-level resistant mutants were selected in a step-wise manner with moxifloxacin. The QRDRs of *gyrA* and *grlA* were characterised by DNA sequencing. Although all five first-step mutants had elevated MICs to moxifloxacin, only one was found to have a Ser80 to Phe mutation in *grlA*. No other mutations were detected in any other first-step mutants. All second-step mutants were found to have a *gyrA* mutation, and two strains also had an inherited *grlA* mutation. However, the strains with two mutations did not have higher MICs of moxifloxacin than other second-step mutants. High level resistance in third-step mutants was conferred by a *grlA* mutation in addition to the inherited *gyrA* mutation. Subsequent mutant selections with ciprofloxacin gave similar results with a single first step strain showing a *grlA* mutation, while several second-step mutants had only *gyrA* mutations. These results indicate DNA gyrase and not topoisomerase IV as the primary target of moxifloxacin in *S. aureus*. No mutations could be detected in the region of *gyrB* associated with quinolone resistance in first-step mutants. Uptake and accumulation assays did not show enhanced efflux of moxifloxacin, indicating that low-level moxifloxacin resistance in these strains is facilitated by an unknown mechanism of resistance.

The activity and selection of resistance *in vivo* was investigated in a murine subcutaneous abscess model. Moxifloxacin was found to kill *S. aureus* in subcutaneous abscesses in a dose-dependent manner. *In vivo* mutants were selected

from subcutaneous abscesses and characterised by PCR amplification and DNA sequencing. One first step mutant had a *grlA* mutation but no other mutations were apparent within the QRDRs in first step mutants; the second step mutant had a *gyrA* mutation but no *grlA* mutation. The findings of this thesis are in contrast to those of previous researchers and indicate that the development of resistance and identification of the primary target of quinolones in *S. aureus* may be strain, drug or method dependent and not as simple as previous research has suggested. The relationship between these data and the development of high-level quinolone resistance in clinically significant *S. aureus* strains remains unclear.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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Dedication

This thesis is dedicated to mum and dad for encouraging me to believe you can achieve anything you set your mind to if you take it one step at a time.

Publications and Presentations

Durham, E.J., Amyes, S.G.B., Dalhoff, A. & Thomson, C.J. (1999) Mechanism of activity of moxifloxacin against *Staphylococcus aureus in vitro*. *Moxifloxacin in Practice* **1**: 57-62.

Durham, E.J., Thomson, C.J., Dalhoff, A. & Amyes, S.G.B. (1999) Preferred targets of moxifloxacin in *Staphylococcus aureus*. *Moxifloxacin in Practice* **1**: 63-69.

Amyes, S.G.B., Durham, E.J., Thomson, C.J. & Dalhoff, A. (1999) Enhanced efficacy of moxifloxacin against *Staphylococcus aureus*. Abstract 079. *9th European Congress of Clinical Microbiology and Infectious Diseases*, Berlin.

Durham, E.J., Amyes, S.G.B., Dalhoff, A. & Thomson, C.J. (1999) Moxifloxacin does not promote resistance during treatment of staphylococcal abscesses. Abstract P392. *Journal of Antimicrobial Chemotherapy* **44 (Suppl. A),** 127.

Durham, E.J., Thomson, C.J., Dalhoff, A. & Amyes, S.G.B. (1999) High-level resistance mutations suggest that GyrA is the primary target for both ciprofloxacin and moxifloxacin in *Staphylococcus aureus*. *39th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Francisco.

Durham, E.J., Amyes, S.G.B., Dalhoff, A. & Thomson, C.J. (1998) Intracellular targets of moxifloxacin in *Staphylococcus aureus*. Abstract. *6th International Symposium on New Quinolones*, Denver.

Durham, E.J., Amyes, S.G.B., Dalhoff, A. & Thomson, C.J. (1998) *In vivo* activity of moxifloxacin (BAY 12-8039) against *Staphylococcus aureus* in a murine subcutaneous abscess model. Abstract. *6th International Symposium on New Quinolones*, Denver.

Durham, E.J., Amyes, S.G.B., Dalhoff, A. & Thomson, C.J. (1997) *In vitro* activity of BAY 12-8039 against *Staphylococcus aureus*. Abstract F-139. *37th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Toronto.

Abbreviations

ADP	-	adenosine di-phosphate
ATP	-	adenosine tri-phosphate
BHI	-	brain heart infusion broth
bp	-	base pair
CCCP	-	carbonyl cyanide m-chlorophenyl hydrazone
CNS	-	central nervous system
DNA	-	deoxyribonucleic acid
FDA	-	Food and Drug Administration, USA
MIC	-	minimum inhibitory concentration
MHA	-	Mueller Hinton agar
MRSA	-	methicillin-resistant *Staphylococcus aureus*
MSSA	-	methicillin-sensitive *Staphylococcus aureus*
NB	-	nutrient broth
OBC	-	optimum bactericidal concentration
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
QRDR	-	quinolone resistance determining region
Rep-PCR	-	repetitive extragenic palindromic-PCR

The standard single and three letter abbreviations are used for the amino acids.

Contents

Chapter 1: Introduction

1.1 Antibiotic Resistance

The discovery of antibiotic compounds in the early part of this century led to a revolution in the treatment of infectious diseases. So successful were antibiotics, that in 1969 the Surgeon General of the United States of America addressed Congress regarding the efficacy of antibiotics with the following statement "The time has come to close the book on infectious disease". Unfortunately this statement was premature, as bacteria have continued to show themselves to be adept at overcoming antimicrobial challenges with which they are faced. In fact today up to 40% of *Staphylococcus aureus* strains in USA and 80% in China are resistant to not just one, but multiple antibiotics (Williams, 1999).

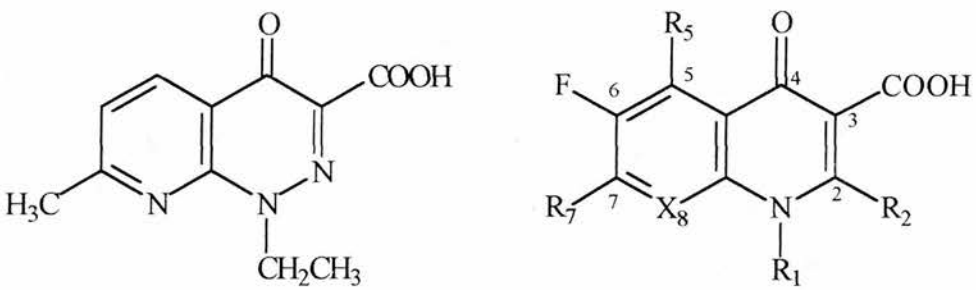
1.2 Quinolone Antibiotics

The creation of new antibacterial agents is fraught with difficulties, and the majority of agents in use are still natural microbial products rather than synthetic chemicals. One of the first synthetic antibiotics, nalidixic acid, was discovered as a by-product of the purification process of the anti-malarial drug chloroquine (Lesher *et al.* 1962). Nalidixic acid was the first in a new class of antibacterial agents known as the quinolones. It showed good activity against certain Gram-negative organisms, notably the *Enterobacteriaceae* (Lesher *et al.* 1962), but had only poor activity against *S. aureus* and other Gram-positive bacteria. This limited spectrum of activity

combined with poor serum and tissue concentrations, when given *in vivo*, restricted the clinical applications of nalidixic acid to the treatment of urinary tract infections.

It took almost 20 years of research and development into derivatives and structural analogues before the breakthrough of incorporating a fluorine atom at position C-6 of the quinolone core structure led to the construction of fluoroquinolones, providing a new spectrum of clinically useful drugs with superior activity to nalidixic acid. Figure 1.1 shows the structure of nalidixic acid and the fundamental fluoroquinolone structure.

Figure 1.1: Nalidixic acid and fluoroquinolone core



a) Nalidixic acid

b) Fluoroquinolone core

1.3 Structure-Activity Relationships

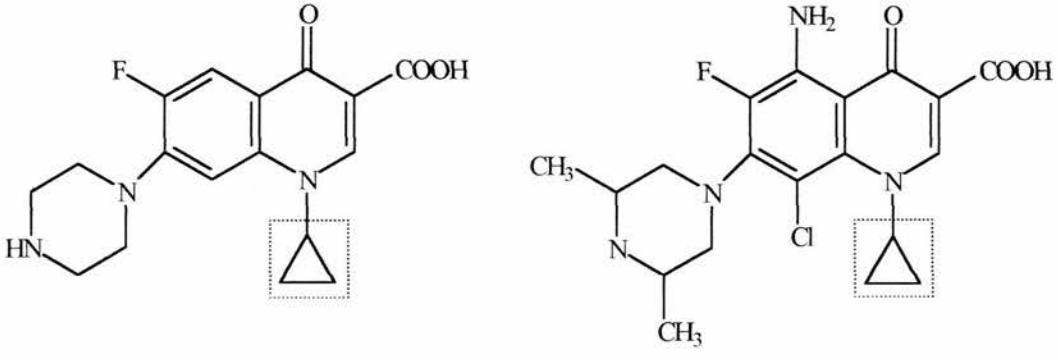
Today more than 10 000 analogues of nalidixic acid and the fluoroquinolones have been described, although very few have been licensed for clinical use. However, this abundance of compounds has facilitated study of the structure-activity relationships between quinolones and their applications both *in vitro* and *in vivo*. Such studies

have revealed some correlation between substituents at key positions within the quinolone core structure and the bactericidal spectrum, *in vivo* toxicity and, most recently, resistance potential of these agents (Zhao *et al.* 1998; Tillotson, 1996).

1.3.1 Position 1

The fundamental quinolone core structure is shown in Figure 1.1b, with all possible sites of modification labelled numerically. The substituent at position 1 controls the overall antimicrobial potency of the agent and is optimally a cyclopropyl group as found in ciprofloxacin (Figure 1.2a) and sparfloxacin (Figure 1.2b) giving significant activity against Gram-negative bacteria such as *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Tillotson, 1996). The sterical, spacial and electron donation effects of larger moieties at this position can have an adverse influence on the biological activity (Chu and Fernandes, 1989), although substitution of a 2,4-difluorophenyl group may enhance activity against anaerobic bacteria (Domagala, 1994).

Figure 1.2: Ciprofloxacin and sparfloxacin

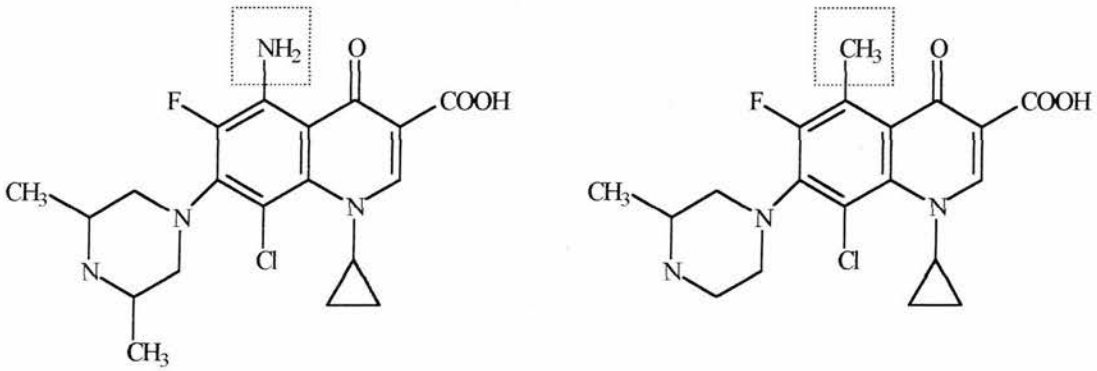


a) Ciprofloxacin b) Sparfloxacin

1.3.2 Positions 2 - 6

Few changes at positions 2, 3 and 4 have been investigated primarily because addition of large substituents causes steric hindrance preventing the interactions of the carboxyl group at position 3 and the oxygen molecule at position 4 with the bacterial DNA/DNA gyrase complex. Such a hindrance substantially decreases the antibacterial activity of the compound (Domagala, 1994).

Figure 1.3: Sparfloxacin and grepafloxacin



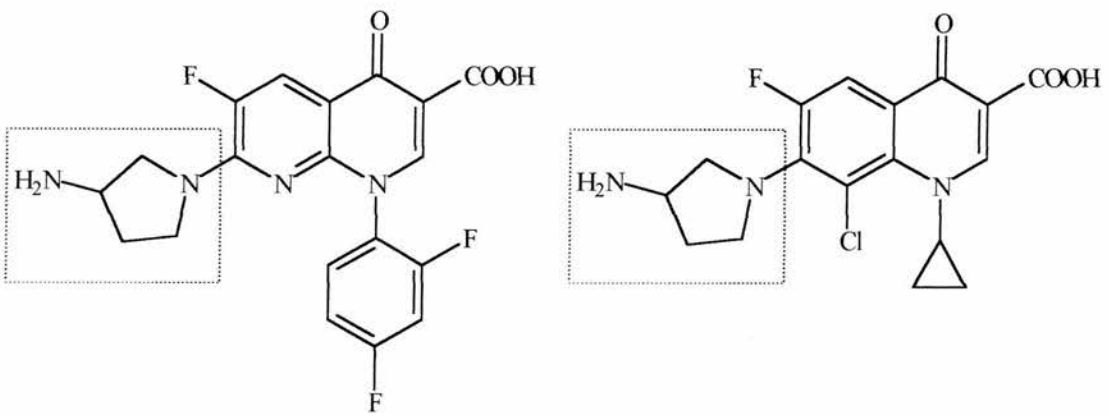
a) Sparfloxacin b) Grepafloxacin

Alterations at position 5 have not been extensively studied. However, newer agents such as sparfloxacin (Figure 1.3a) and grepafloxacin (Figure 1.3b) with additional moieties at this position have enhanced activity against Gram-positive organisms compared to earlier agents. The addition of small highly active atoms, particularly fluorine, at position 6 generate a significant increase in activity possibly by enhancing cell penetration and binding to the bacterial DNA/DNA gyrase complex (Chu and Fernandes, 1989).

1.3.3 Position 7

Alterations at position 7 have been extensively studied and a range of different substituent groups alter the spectrum of activity. Molecules with large cyclic groups, particularly those incorporating a nitrogen atom, have increased antibacterial activity and better pharmacokinetic profiles (Tillotson, 1996).

Figure 1.4: Tosufloxacin and clinafloxacin

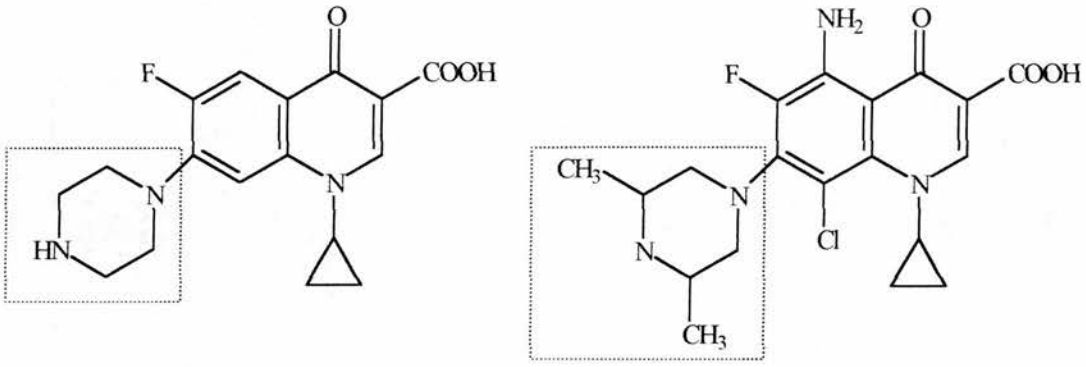


a) Tosufloxacin

b) Clinafloxacin

The aminopyrrolidine group of tosufloxacin (Figure 1.4a) and clinafloxacin (Figure 1.4b) enhances anti-Gram-positive activity. Ciprofloxacin (Figure 1.5a) has a piperazine group at this position, enhancing anti-Gram-negative activity. Similarly, sparfloxacin (Figure 1.5b) has a piperazine group enhancing anti-Gram-negative activity, but the additional alkylation also enhances anti-Gram-positive activity and serum half-life.

Figure 1.5: Ciprofloxacin and sparfloxacin

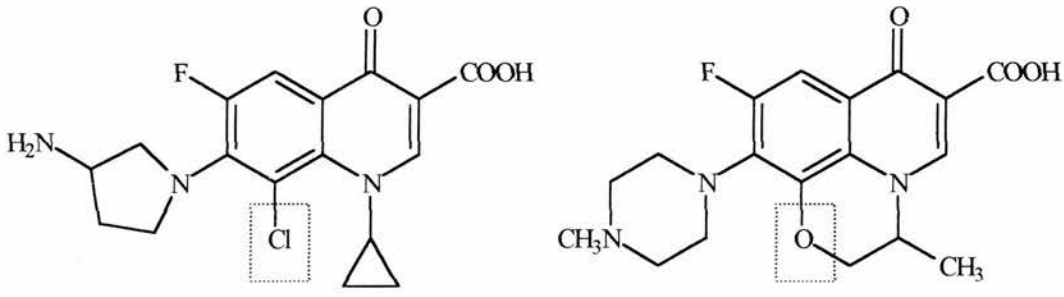


a) Ciprofloxacin b) Sparfloxacin

1.3.4 Position 8

Substitution of carbon at position 8 with C-8-chloro (Figure 1.6a) or C-8-fluoro may produce derivatives with greater activity *in vivo* due to enhanced absorption, but which are often less active *in vitro* (Chu and Fernandes, 1989).

Figure 1.6: Clinafloxacin and ofloxacin



a) Clinafloxacin b) Ofloxacin

Ofloxacin (Figure 1.6b) has oxygen at position 8 that has been connected with good *in vivo* efficacy (Chu and Fernandes, 1989).

1.4 Structure-Toxicity Relationships

Predicting the activity of structural analogues and derivatives of quinolones is difficult because of the risk of producing compounds with high *in vivo* toxicity. For example, changes that enhance one aspect of quinolone activity, such as *in vitro* activity, may have a detrimental effect on the pharmacokinetic profile (Domagala, 1994). Quinolones can cause a number of toxic effects *in vivo* including gastrointestinal symptoms, skin rash, phototoxicity, arthropathy, crystalluria and CNS effects (Domagala, 1994). Some *in vivo* toxicity can be associated with position-specific structural features of quinolone molecules. For example, phototoxicity has been linked to substitution of halogens at position 8 (Man *et al.* 1999; Tillotson, 1996). Phototoxicity is an oxygen-dependent non-immunogenic reaction caused by the decay of the quinolone molecule after exposure to UVA radiation. Products of this decay process include singlet oxygen and radicals which can cause severe damage to tissues (Domagala, 1994). This form of toxicity has been serious enough to warrant discontinuation of development of many compounds such as BAY 3118 that otherwise showed great potential based on *in vitro* activity (Tillotson, 1996).

Although side effects may be rare, they can be sufficiently serious as to severely compromise the therapeutic value of the drug. Trovafloxacin, for example, is one of the newer quinolones with good activity against Gram-positive bacteria such as *S. aureus* and respiratory pathogens. It was licensed in the USA in December 1987, where it has been on the market since February 1998. In clinical trials of

approximately 7000 patients prior to licensing there were no reports of serious adverse hepatic reactions. However, in July the FDA issued a public health advisory indicating that at least 14 cases of acute liver failure, six of which were fatal, had been reported which were strongly associated with use of trovafloxacin (Nightingale, 1999). Although the drug has not been withdrawn, the FDA recommends that patients for whom this drug is being considered should meet stringent criteria to ensure that the benefit for the patient sufficiently outweighs the potential risk. Therefore it seems unlikely that this drug will be routinely prescribed in the future. Similarly, in a press release in October, Glaxo Wellcome voluntarily withdrew grepafloxacin after concerns over "a small number of severe cardiovascular events" which suggested that the risk to patients outweighed the benefits of therapy (Glaxo Wellcome, 1999).

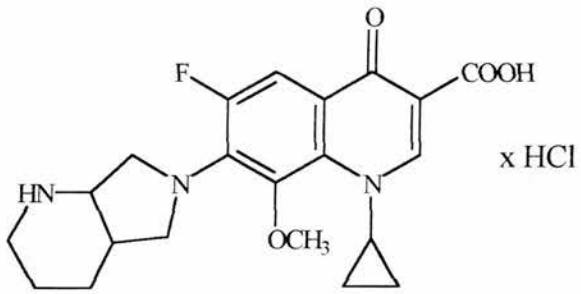
1.5 Moxifloxacin

A notable improvement in recent years has been the development of quinolones that have enhanced activity against Gram-positive organisms, an area of efficacy in which the majority of earlier compounds were lacking. BAY 12-8039, known as moxifloxacin (Figure 1.7), is a new generation fluoroquinolone that has enhanced activity against Gram-positive organisms compared to older agents such as ciprofloxacin (Alcala *et al.* 1999; Balfour and Wiseman, 1999; Dalhoff *et al.* 1996). Moxifloxacin has a cyclopropyl group at position 1, which in ciprofloxacin and sparfloxacin is associate with activity against *Enterobacteriaceae* and *Pseudomonas aeruginosa.* It also has oxygen at position 8, which in ofloxacin has been connected

with good *in vivo* efficacy. Moxifloxacin has a large R_7 group, which in sparfloxacin and ciprofloxacin has been linked to better biological activity. Within this group, nitrogen is required for oral efficacy, and ring alkylation enhances Gram-positive activity and pharmacokinetics, including serum half-life.

Figure 1.7: Moxifloxacin

Systematic name: 1-cyclopropyl-7-([S,S]-2,8-diazabicyclo[4.3.0]non-8-yl)-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolincarboxylic acid hydrochloride



Early activity and pharmacokinetic studies of moxifloxacin confirm some of these theoretical predictions of activity based on structure-activity studies of earlier quinolones. Moxifloxacin has a broad spectrum of activity against both Gram-negative and Gram-positive organisms, although activity against *Pseudomonas aeruginosa* is lower than might be predicted based on the molecular structure (Dalhoff *et al.* 1996). At a single dose of 400mg per day it penetrates well into tissues and rarely causes adverse CNS or phototoxic effects, although gastrointestinal side effects are more common (Balfour and Wiseman, 1999; von Keutz and Schlüter, 1999).

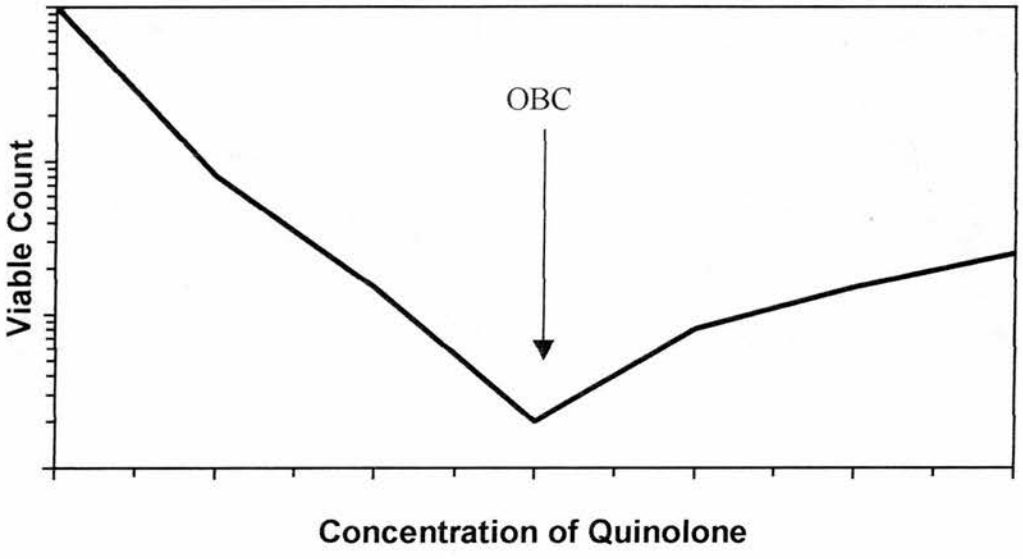
1.6 Bactericidal Activity of Quinolones

Although quinolones have been available clinically for almost two decades their mechanisms of bactericidal activity are still not fully understood. Some features are easily observed microscopically. For example, they cause a number of phenotypic changes including cell filamentation and loss of septation by preventing the completion of cell division (Maxwell and Critchlow, 1998). Other aspects requiring analysis by molecular techniques are discussed in section 1.7.4.

1.6.1 Dose-Response and Optimum Bactericidal Concentration

In the early 1980s Smith demonstrated the 'classic' bactericidal activity pattern of the quinolones – the biphasic dose-response curve shown in Figure 1.8. He demonstrated that although the number of surviving cells decreased as the concentration with which challenge was made increased, there was a minimum survival point (indicated on figure 1.8) at a fixed concentration after which the number of surviving cells appeared to increase again. This minimum concentration was termed the most bactericidal concentration, later called the optimum bactericidal concentration (OBC) (Lewin and Smith, 1990). It has been demonstrated for a number of quinolones including nalidixic acid, norfloxacin and ciprofloxacin and others (Lewin *et al.* 1989; Lewin and Smith, 1988; Ratcliffe and Smith, 1985; Ratcliffe and Smith, 1984; Smith, 1984; Crumplin and Smith, 1975).

Figure 1.8: Biphasic Dose Response Curve



Smith suggested that the increased survival of cells challenged with concentrations greater than the OBC resulted from the excessively high concentrations of quinolone inhibiting bacterial RNA synthesis, a process thought to be crucial to the bactericidal action of quinolones (Crumplin and Smith, 1975). Interestingly the OBC does not directly relate to the minimum inhibitory concentration (MIC) at which concentration quinolones are merely bacteriostatic (Smith and Zeiler, 1998).

1.6.2 Mechanisms of Action

While investigating the effects of protein and RNA synthesis inhibitors, Smith was able to demonstrate the possibility of various mechanisms of action of quinolones. The simultaneous challenge of a broth culture with both bactericidal concentrations of quinolone and a bacteriostatic concentration of an inhibitor such as rifampicin (or chloramphenicol in the case of Gram-positives) investigated the possibility of

quinolone activity in the absence of RNA synthesis. Similarly, incubation of a culture challenged with quinolone in a minimal medium, such as phosphate buffered saline (PBS), with a concomitant lack of available energy resources, investigated the activity of drug when cells were not actively growing. Based on such experiments, Smith (1984) developed the hypothesis of three mechanisms of bactericidal activity possibly exhibited by quinolones. Mechanism A, demonstrated by all quinolones, is exhibited against actively growing cells and can be lost by addition of inhibitors or by prevention of active cell division. It is the only mechanism of older quinolones such as nalidixic acid (Lewin *et al.* 1991b). Mechanism B, however, can be demonstrated irrespective of active cell division and does not require active RNA synthesis (Smith, 1984). Mechanism B has been found in addition to mechanism A for a number of fluorinated quinolones including ciprofloxacin, ofloxacin (Ratcliffe and Smith, 1984), lomefloxacin (Lewin *et al.* 1989) and sparfloxacin (Lewin *et al.* 1992). An additional mechanism B^1 described for clinafloxacin (Lewin and Amyes, 1990) requires active cell division but is independent of active protein or RNA synthesis. Finally, mechanism C does require protein and RNA synthesis but not actively growing cells. This mechanism has only been detected with norfloxacin and enoxacin in *E. coli* (Lewin *et al.* 1989; Ratcliffe and Smith, 1985). The four mechanisms are summarised in Table 1.1. Not all quinolones are found to have the same bactericidal mechanisms against all species. The relationship between mechanisms of action and activity is somewhat complex; mechanisms of action seem to be quinolone, organism and concentration specific. For example, both ciprofloxacin and ofloxacin kill *E. coli* by mechanisms A and B. However,

ciprofloxacin is a much more active compound against this species. Conversely, both drugs have similar activity against *S. aureus*, although ofloxacin kills by both mechanisms A and B while ciprofloxacin apparently only exhibits mechanism A (Lewin and Smith, 1988). Mechanism B seems to be of key importance to the activity of these two quinolones. For example they have poor activity against *E. faecalis* and *S. pneumoniae* against which they demonstrate no or only weak killing by mechanism B (Morrissey and Smith, 1993; Lewin *et al.* 1991a).

Table 1.1: Requirements for mechanisms of quinolone action

Mechanism	Protein/RNA Synthesis?	Active Cell Division?
A	yes	yes
B	no	no
B^1	no	yes
C	yes	no

It is still unclear exactly how these killing mechanisms, and the concentrations at which they begin to be manifested, relate to intracellular quinolone targets and binding. However, some evidence seems to correlate the concentration of norfloxacin required to inhibit DNA supercoiling (Shen *et al.* 1989a) with the concentration at which mechanism C is initiated (Smith and Zeiler, 1998). Very little work has so far been done to investigate any effects of specific quinolone resistance mutations on the mechanisms of quinolone killing action.

1.7 Bacterial Topoisomerases

Within a living bacterial cell DNA exists in a dynamic equilibrium of positive and negative superhelical twists controlled by topoisomerases. Bacterial topoisomerases alter the topology of DNA by breaking and passing DNA strands. This, in turn, brings about changes to the tertiary DNA structure by introducing or removing superhelical twists (Cozzarelli, 1980). This has implications for DNA replication, a process dependent on the degree of DNA supercoiling. There are two classes of topoisomerase: Type I, which includes topoisomerase I and topoisomerase III, can break single strands of a double stranded DNA complex, then pass a single strand through the break and reseal the incised portion. This process relaxes the negative superhelical twists in DNA (i.e. introduces positive supercoils). In contrast, type II topoisomerases such as DNA gyrase and topoisomerase IV break both strands of double stranded DNA, and then pass double stranded DNA through before resealing the break (Maxwell and Critchlow, 1998). Activity inhibition studies of DNA gyrase and topoisomerase IV, where binding of the drug reduces enzyme activity, have shown that both these enzymes are targets of the quinolones (Ferrero *et al.* 1994; Gellert *et al.* 1977). Evidence that quinolone resistance mutations, described later in section 1.9, map to the genes encoding DNA gyrase and topoisomerase IV also indicates that these are quinolone targets (Everett and Piddock, 1998).

1.7.1 DNA Gyrase

DNA gyrase (known as topoisomerase II in mammalian cells) is the only topoisomerase able to introduce negative supercoils into DNA (Gellert *et al.* 1977; Gellert *et al.* 1976). This means that it is able to twist DNA so that it is underwound relative to linear DNA. The action of DNA gyrase is counterbalanced by topoisomerase I, which is able to remove these twists thereby maintaining and adjusting the superhelical density of DNA (Hooper, 1998). By adding negative supercoils ahead of the replication fork, and at sites of transcription by RNA polymerase, DNA gyrase is able to maintain the integrity of DNA replication and transcription by preventing inhibitory levels of supercoiling from accumulating (Hooper, 1998).

1.7.2 Topoisomerase IV

Topoisomerase IV, which shows considerable homology to DNA gyrase, can also remove negative and positive superhelical twists. It is a more efficient decatanase than DNA gyrase (Hoshino *et al.* 1994), and is particularly efficient at removing circles of interlocked or catenated DNA. It is the principal enzyme involved in releasing newly replicated DNA from the interlocked position with the parent DNA, enabling daughter chromosomes to segregate into daughter cells (Khodursky *et al.* 1995; Adams *et al.* 1992). To some extent DNA gyrase and topoisomerase are able to compensate for each other if an activity deficiency is present in one enzyme (Kato *et al.* 1992; Kato *et al.* 1990).

Both DNA gyrase and topoisomerase IV are tetrameric enzymes composed of two A and two B subunits (Drlica and Zhao, 1997). The *gyrA* and *gyrB* genes encode these subunits respectively for DNA gyrase. In *S. aureus*, *grlA* and *grlB* encode the subunits of topoisomerase IV, and are equivalent to *parC* and *parE* in *Escherichia coli*.

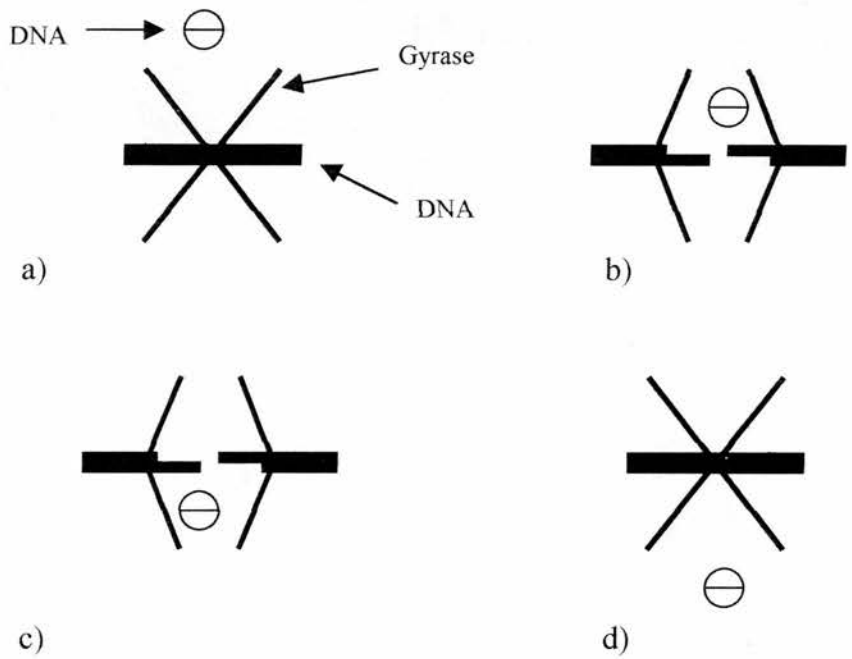
1.7.3 Interactions of DNA Gyrase and Topoisomerase IV with DNA

Taking DNA gyrase as an example, the interaction between the enzyme and DNA at a molecular level can be described as follows. The A subunit interacts directly with DNA via the tyrosine residue at position 122 which constitutes the active site and is located within the highly conserved N-terminal region of the protein (Maxwell and Critchlow, 1998). Hydrolysis of ATP to ADP by the B subunit provides sufficient energy to drive the supercoiling activity of the enzyme (Drlica and Zhao, 1997). It also facilitates enzyme turnover, restoring the tertiary enzyme conformation to a ready state for subsequent catalytic activity. Clearly therefore the ratio of ATP to ADP can exert some degree of control on the level of DNA supercoiling. Since this ratio is influenced by external environmental factors such as salt concentration (Hsieh *et al.* 1991), external factors can directly influence genomic activity. Homeostatic regulation has also been described in which a decrease in supercoiling, possibly caused by the presence of inhibitors, upregulates expression of DNA gyrase thereby increasing supercoiling levels again (Menzel and Gellert, 1983).

The interaction of DNA gyrase and DNA appears to form a transiently stable cleavage complex (Reece and Maxwell, 1989), although the exact details of how this

occurs have yet to be clarified. Figure 1.9 shows a schematic diagram of the DNA gyrase/DNA interaction (Drlica and Zhao, 1997).

Figure 1.9: DNA gyrase introduces negative supercoils into DNA



Adapted from Drlica and Zhao (1997)

The A subunit of DNA gyrase binds to DNA (Figure 1.9a), resulting in a region of approximately 130 base pairs of DNA wrapping around the DNA gyrase molecule (Maxwell and Critchlow, 1998). The B subunit then binds ATP causing a change in the tertiary conformation of the DNA gyrase molecule (Figure 1.9b). This structural adjustment breaks the double strands of DNA in a four base stagger, effectively pulling the double stranded DNA apart to form a gate (Morrison and Cozzarelli, 1979). A temporary covalent bond is formed between the 5′ phosphate of DNA and the Tyr122 active site of GyrA (Horowitz and Wang, 1987). A double strand of

DNA is then passed through this open gate (Figure 1.9b and c), and the DNA break is closed (Figure 1.9d). Hydrolysis of ATP to ADP by GyrB completes turnover of the enzyme restoring it to the relaxed conformation and freeing it ready to bind again as required.

1.7.4 Interactions of Quinolones with DNA Gyrase and Topoisomerase IV

Quinolones are thought to bind to the cleavable DNA-gyrase complex and prevent the resealing of double stranded breaks in DNA. This in turn initiates the SOS response (Hooper, 1998), inducing expression of several genes responsible for repair of damaged DNA or lesions in DNA replication (Walker, 1984). Quinolone inhibition of the cleavable complex thus inhibits DNA replication and transcription. Critchlow and Maxwell (1996) showed that the normally transient GyrA-GyrB-DNA complex is stabilised by the addition of a quinolone, but exactly how this occurs is still unclear. The interaction process with topoisomerase IV is thought to be similar as quinolone binding inhibits DNA decatenation and relaxation in a similar manner (Ng *et al.* 1996; Chen *et al.* 1996; Khodursky *et al.* 1995; Ferrero *et al.* 1994; Hoshino *et al.* 1994; Kato *et al.* 1992). Many methods of study have been used to examine the binding of quinolones to cleavable complexes. However, the results have varied depending on the methodology employed (Orphanides and Maxwell, 1994; Rau *et al.* 1987; Fisher *et al.* 1981; Morrison and Cozzarelli, 1981) and a clear consensus on the exact mechanism has yet to be reached. At the present time it seems that the only conclusion to be drawn is that quinolones trap the cleavable

complexes of the enzyme by binding and blocking after or immediately before the double stranded DNA break is incurred.

1.8 Drug-DNA-enzyme binding models

Although it is not clear at what point the quinolone interacts with the DNA gyrase-DNA complex, certain features of binding have been determined. Work by Willmott and Maxwell (1993) indicated that ^{3}H-labelled norfloxacin does not bind to DNA gyrase or DNA alone, although significant binding to a complex of both DNA gyrase and DNA can be detected (Yoshida *et al.* 1993). Conversely, Shen *et al* (1989a) were able to detect quinolone binding to relaxed DNA alone, although binding was increased if gyrase was present. In the light of these apparently contradictory results several drug-binding models have been suggested.

1.8.1 The Cooperative Drug Binding Model

The first of these is the cooperative drug-binding model of Shen *et al* (1989b) shown in Figure 1.10. DNA gyrase, with A and B subunits, is indicated by dotted outlines. Four stacked quinolone molecules are shown as filled and hatched boxes in the center of the figure. In this hypothesis, DNA gyrase binds to DNA and cleaves it in an ATP-dependent process leaving a four base stagger of single-stranded DNA at the DNA gyrase active site.

Figure 1.10: Cooperative drug binding model

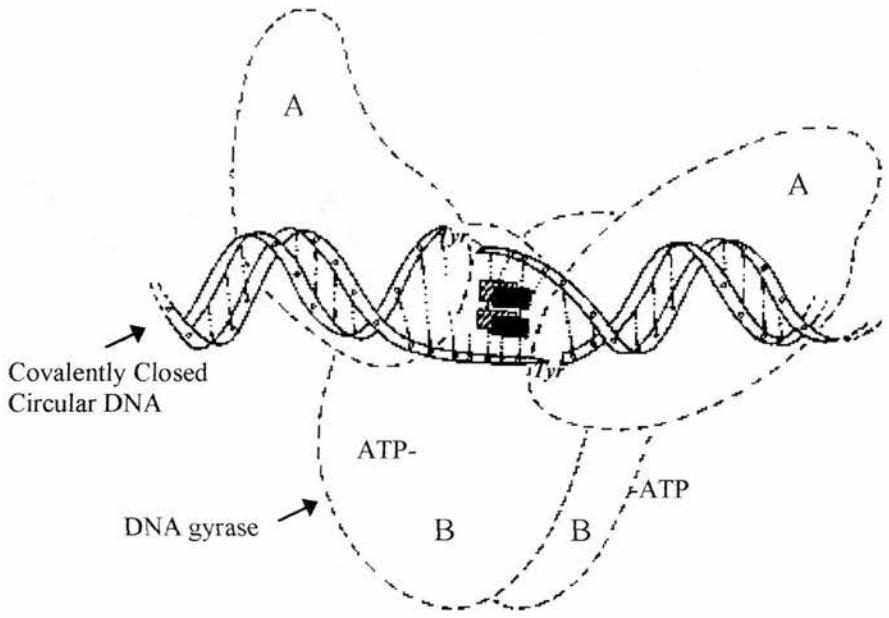
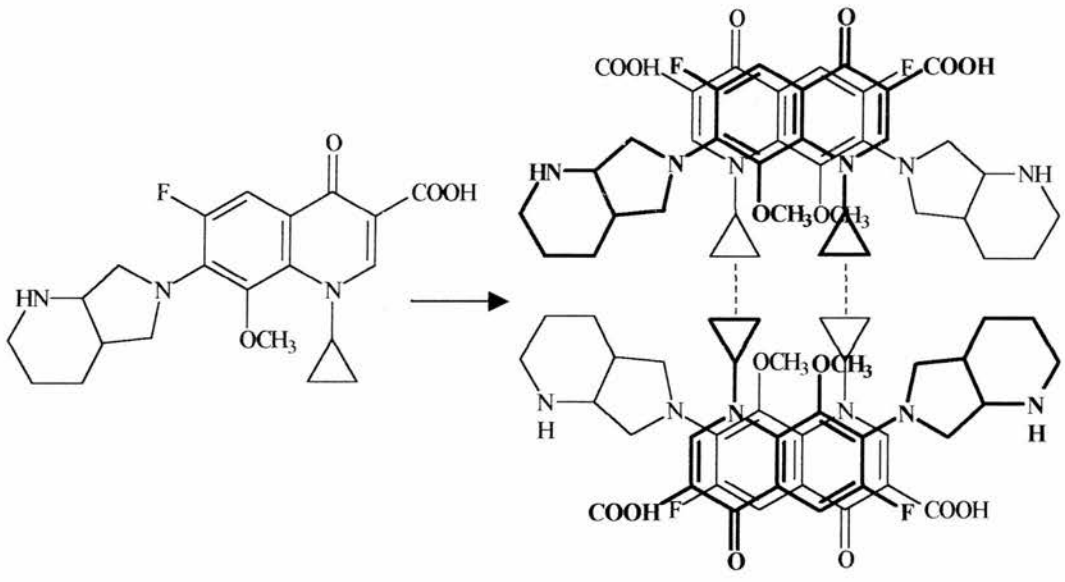


Figure taken from Shen *et al* (1989b)

Figure 1.11: Quinolone molecule stacking in cooperative drug binding model



a) Single moxifloxacin molecule b) Four stacked moxifloxacin molecules

Four quinolone molecules stack as shown in Figure 1.11, and bind to the exposed DNA strands via hydrogen bonds between single stranded DNA donors and the carbonyl and carboxyl groups at positions 3 and 4 on the quinolone nucleus. One problem with this model is that it requires cleavage of DNA; however quinolones have been shown to bind to complexes formed with DNA gyrase mutants that do not have a Tyr122 active site implying that DNA cleavage is not actually necessary for quinolone binding (Critchlow and Maxwell, 1996).

1.8.2 The Magnesium Bridge Model

A second model known as the magnesium bridge model has been suggested (Figure 1.12). Binding of quinolones to single stranded non-linear DNA is known to be Mg^{2+} dependent (Palù *et al.* 1992; Palù *et al.* 1988).

Figure 1.12: Mg^{2+} bridge model

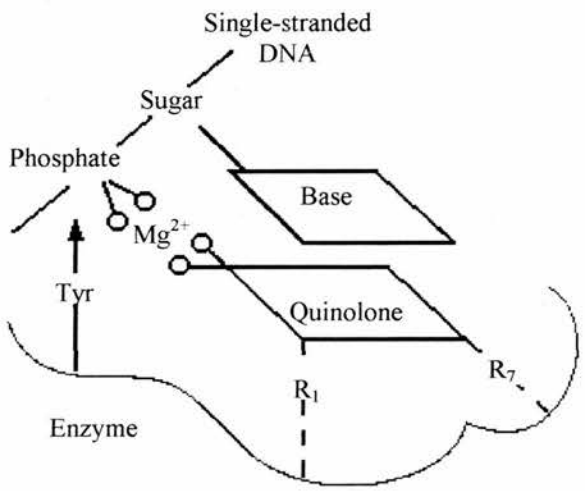


Figure taken from Palumbo *et al* (1993)

In this model is it proposed that Mg^{2+} forms a bridge between the phosphate of the nucleic acid and the carbonyl and carboxyl groups of the quinolone, while the C-7 and N-1 quinolone substituents interact with DNA gyrase. Clearly the Mg^{2+} concentration is crucial to this process as insufficient magnesium would prevent bridge formation, while an excess would saturate both the DNA and the quinolone. As with the previous hypothesis, this model requires binding of quinolone to single stranded DNA produced by the cleavage process, however the presence of cleaved duplex DNA has been shown to be irrelevant to quinolone binding (Critchlow and Maxwell, 1996).

Both the models described above assume that the C-7 substituent of a quinolone interacts with gyrase, but this seems highly unlikely given the vast diversity of such substituents (see section 1.3).

1.8.3 Other Binding Models

Several other models with less supporting evidence have been proposed. The pocket binding model suggested by Yoshida *et al* (1993) supposes that quinolones bind in a pocket of the gyrase-DNA complex that appears during the DNA cleavage and reunion process, and that quinolone binding affinity is determined by both the GyrA and GyrB subunits of the enzyme. Mutations could therefore alter the shape of the pocket preventing efficient binding. Another model, presented by Fan *et al* (1995), is based on the intercalation of quinobenzoxazines. These drugs are structural analogues of norfloxacin, which inhibit mammalian topoisomerase II although they

have no antibacterial activity. This model also presupposes a dependence on Mg^{2+} as a bridge between the drug and DNA molecules as suggested in other models. However, it is based on intercalation of the drug into the DNA double helix structure, something which has been discounted by other studies (Shen and Pernet, 1985).

Many questions remain to be answered before a more accurate model can be presented. At the present time it is not clear which amino acids in DNA gyrase and topoisomerase IV are actually involved in quinolone binding. The constituents of the quinolone molecule, that interact with DNA gyrase or topoisomerase IV, and the manner in which they do so must also be determined. If these basic aspects of quinolone action can be clarified, a more accurate hypothesis of how quinolones, DNA and topoisomerases interact may be formulated. At the present time it may only be concluded that quinolones bind to cleavable complexes by a process in which Mg^{2+} plays a key role.

1.9 Mechanisms of Quinolone Resistance

Bacteria have an intrinsic potential for genetic change and hence mutations, which allow them to overcome antibiotic challenge, frequently arise. Three mechanisms of quinolone resistance have been described so far in *S. aureus*: mutations in *gyrA* and *gyrB*, the genes encoding the A and B subunits of DNA gyrase; mutations in *grlA* and *grlB*, the equivalent genes that encode topoisomerase IV; altered expression of transmembrane efflux proteins. Resistance to the quinolones is chromosomal rather than plasmid encoded and, in the early years of quinolone development, this gave

rise to speculation that quinolone resistance would not develop as a serious clinical problem. Although few clinically significant pathogens such as methicillin-sensitive *S. aureus* (MSSA), *E. coli*, and *Klebsiella* species in the UK currently show any quinolone resistance (Schmitz *et al.* 1999), the number of reported quinolone resistant strains isolated globally is slowly increasing (Thomson, 1999). In addition, a recent report (Martinez-Martinez *et al.* 1998) has shown that it is possible to transfer quinolone resistance by plasmid vector, giving the potential for rapid spread of resistance in the future.

1.9.1 Mutations in DNA Gyrase

A number of point mutations occur within the *gyrA* and *gyrB* genes of DNA gyrase that reduce sensitivity to quinolones. Since there is a high degree of homology between different bacterial species, these mutations map to equivalent positions on most bacterial genomes. In *gyrA* a number of mutational hotspots, so called because they have a greater propensity for mutation that other codons, have been identified within a region of approximately 300 base pairs. This gene segment has been termed the quinolone resistance determining region (QRDR) (Yoshida *et al.* 1990a). The most common sites of mutation in the QRDR of *S. aureus* are at codons 84, 85 and 88 (equivalent to 83, 84 and 97 in *E. coli*), and numerous point mutations have been described in both laboratory strains and clinical isolates (Schmitz *et al.* 1998; Takahashi *et al.* 1998; Fitzgibbon *et al.* 1998; Wang *et al.* 1998; Deplano *et al.* 1997; Ferrero *et al.* 1995; Ito *et al.* 1994; Nakanishi *et al.* 1991; Sreedharan *et al.* 1990). Similar mutations at equivalent positions have been seen, if looked for, in

most other species including *E. coli* (Yonezawa *et al.* 1995; Vila *et al.* 1994; Yoshida *et al.* 1990a) and *Streptococcus pneumoniae* (Varon *et al.* 1999; Pan and Fisher, 1997; Tankovic *et al.* 1996).

GyrB mutations have generally only been described in clinical strains and seem to have a modest effect on sensitivity to quinolones compared to *gyrA* mutations (MunozBellido *et al.* 1999; Schmitz *et al.* 1998; Takahashi *et al.* 1998; Ito *et al.* 1994). Such mutations have been described less frequently than mutations in *gyrA* and thus there is no apparent equivalent to the QRDR of *gyrA* within this gene. However, it is difficult to draw conclusions as to their precise role since most strains in which they have been found also have multiple mutations in *gyrA* and topoisomerase IV which mask the effect of mutations in *gyrB*.

1.9.2 Mutations in Topoisomerase IV

As topoisomerase IV has a high degree of homology to DNA gyrase, it is not surprising that a region analogous to the QRDR of DNA gyrase has also been described in the GrlA subunit of topoisomerase IV (Ferrero *et al.* 1994). In fact in many organisms, including *S. aureus,* the mutation hotspots within *grlA* (*parC* in *E. coli*) map to codons equivalent to those within the QRDR of *gyrA* (ElAmin *et al.* 1999; Deguchi *et al.* 1997; Vila *et al.* 1997; Yamagishi *et al.* 1996; Ng *et al.* 1996; Heisig, 1996; Pan and Fisher, 1996; Ferrero *et al.* 1995; Ferrero *et al.* 1994; Yamagishi *et al.* 1996; Yamagishi *et al.* 1996; Yamagishi *et al.* 1996; Yamagishi *et al.* 1996). The most common *grlA* mutations are in codons 80 and 84 (equivalent to

codons 84 and 88 in *gyrA*). Mutations within *S. aureus grlB* have rarely been described and confer only a small reduction in sensitivity to quinolones (Schmitz *et al.* 1998; Tanaka *et al.* 1998). Therefore it appears that, as with *gyrB*, mutations within this gene may be of lesser importance to clinical quinolone resistance.

1.9.3 Resistance by Enhanced Efflux

Several multi-drug efflux pumps have been described in a number of bacterial species. The NorA pump described in *S. aureus* (Trucksis *et al.* 1991; Yoshida *et al.* 1990b; Ubukata *et al.* 1989) is a chromosomally encoded active efflux pump requiring proton motive force, and is not a passive protein channel (Kaatz *et al.* 1993). It is inherently expressed in wild-type cells but exhibits only a very low level of quinolone efflux, showing a preference for hydrophilic quinolones such as norfloxacin (Piddock *et al.* 1999b; Kaatz *et al.* 1993; Mortimer and Piddock, 1991). Such efflux pumps are transmembrane proteins that have broad substrate specificity i.e. they are able to export a variety of different molecules from the bacterial cell, and are not restricted to efflux of quinolones alone. Although the natural role of NorA has yet to be determined, it shows a high degree of sequence similarity to the Bmr protein from *Bacillus subtilis* (Yoshida *et al.* 1990b). In addition to quinolones, Bmr is known to mediate the efflux of a number of toxins such as ethidium bromide from the cell (Neyfakh, 1992). NorA is also able to efflux a number of structurally diverse compounds (Kaatz and Seo, 1995). In fact, since quinolones are synthetic and not naturally occurring compounds their export by efflux pumps is a fortuitous coincidence for the bacterium. The natural low-level expression of an efflux pump

may have little significant effect on the sensitivity of an organism, unless the wild-type gene is overexpressed thereby having a greater effect in reducing sensitivity to quinolones (Kaatz *et al.* 1993). Overexpression may be induced by pre-exposing the bacterium to a suitable substrate (Kaatz and Seo, 1995), and may be due to a single nucleotide change in the *norA* promoter (Ng *et al.* 1994). Overexpression increases the number of efflux proteins present on the cell surface above wild-type levels of expression, allowing the bacterium to export the antibiotic more rapidly. This may sufficiently reduce the intracellular quinolone concentration so that fatal toxic effects are prevented and survival time increases. Extending the survival period after antibiotic challenge may allow the cell to develop resistance mutations within gyrase or topoisomerase IV that will enable it to survive subsequent challenges. Resistance by enhanced efflux can be detected by observation of intracellular quinolone accumulation. The introduction of inhibitors such as the plant alkaloid reserpine, or protonophores such as CCCP into a broth culture can significantly increase the intracellular quinolone concentration if NorA is being overexpressed (Brenwald *et al.* 1997).

As a resistance mechanism, efflux can only overcome exposure to low levels of quinolone, and it is not clear at what point efflux mutations occur in the overall development of resistance. Some authors have indicated that they may occur early in the progression to high level resistance *in vivo* as an alternative to the development of topoisomerase IV mutations (Sulavik and Barg, 1998).

1.10 Primary and Secondary Quinolone Targets

Resistance to quinolones develops in a step-wise manner. Addition mutations accumulate within DNA gyrase or topoisomerase IV with each new bacterial generation challenged. When topoisomerase IV was discovered as a second target for quinolones in both Gram-negative and Gram-positive organisms, the question as to whether DNA gyrase or topoisomerase IV was the primary quinolone target arose. Mutation studies have indicated that in Gram-negative organisms such as *E. coli*, DNA gyrase is the primary target (Heisig, 1996; Khodursky *et al.* 1995), because mutations in *gyrA* appear before mutations in *parC*. Following the sequencing of *S. aureus* topoisomerase IV by Ferrero *et al* (1994), the development of resistance mutations in *S. aureus* was also investigated. Was there a different primary target in Gram-positive organisms? The QRDRs of *gyrA* and *grlA* of resistant mutants selected with ciprofloxacin in a step-wise manner, were amplified by PCR and sequenced (Ferrero *et al.* 1995). The sequences revealed topoisomerase IV but not DNA gyrase mutations in first-step mutants leading the authors to conclude that topoisomerase IV is the primary target of quinolones in Gram-positive organisms. Subsequently, a number of studies of other bacterial species and clinical isolates confirmed this conclusion (Gonzalez *et al.* 1998; Ng *et al.* 1996; Tankovic *et al.* 1996).

However, this early work was done only with ciprofloxacin, an older quinolone that is primarily active against Gram-negative organisms and has poorer activity against Gram-positive organisms such as *S. aureus* (Al-Nawas and Shah, 1998; Dalhoff *et al.*

1996). Some more recent studies indicate that other factors may also need to be taken into account. For example, it is clear that although cross-resistance is observed throughout the class of quinolone antibiotics, a mutation that confers high level resistance to one quinolone may not confer the same degree of resistance to newer quinolones (Schmitz *et al.* 1998; Takahashi *et al.* 1998; Takahata *et al.* 1996). Recent work also indicates that *gyrA* and *parC* are dual and equal targets in *S. pneumoniae* for sparfloxacin (Pan and Fisher, 1997), clinafloxacin (Pan and Fisher, 1998) and several other quinolones (Varon *et al.* 1999), rather than one enzyme being designated as the primary target. So far little work has been done to investigate this result in other bacterial species with different quinolones.

1.11 Epidemiology of pathogenic S. aureus

The spread of quinolone resistance has been predicted to be limited by the fact that mutations conferring resistance are chromosomal rather than plasmid-encoded (Smith, 1984). This means that a small number of strains may be responsible for clonal spread of resistance since transfer between strains and species is restricted. However, there is still epidemic potential in such a spread if a resistant bacterium such as *S. aureus* has multiple drug and antiseptic resistance genes enhancing survival (Dalhoff, 1994). Indeed, one study found that resistance to perfloxacin, an older quinolone rose as high as 99% in most hospitals in France during 1993 (Goldstein and Acar, 1995). As expected, the rate of resistance has been demonstrated to be lower amongst community acquired isolates compared to nosocomial pathogens that are subjected to more rigorous selective environmental

pressures. However, despite current low levels of resistance the fact remains that resistance to quinolones is increasing globally (Thomson, 1999) so the situation requires continued monitoring and sensible strategies for resistance control.

1.12 A Model for Studying In Vivo Quinolone Activity

Although the activity of all quinolones can be extensively studied *in vitro* it is often difficult to know whether the resulting data is relevant to what happens *in vivo*. Bacteria grown in the artificial, highly controlled environment of the laboratory may not respond in the same way when subject to the rigorous stresses of the human body where mutation pressures may become far greater. Quinolones also may have different activity *in vivo*, where they may react with biological systems and molecules in an unexpected manner. Indeed, the *in vivo* deficiency of oxygen compared to the *in vitro* environment is one factor known to effect the therapeutic activity of quinolones (Smith and Zeiler, 1998). The outcome of treatment cannot therefore be easily predicted unless *in vivo* investigations are made during the research and development stages of a new agent. Although simple dosing models are used to determine preferred routes of administration and pharmacokinetic profile, infection models are also useful. Many animal models have been developed to emulate human infection. One such model is a murine subcutaneous abscess model developed by Bunce *et al* (1992). In this model mice are infected subcutaneously with *S. aureus* to produce abscesses. They are then treated with antibiotic by subcutaneous injection, and the efficacy of antibiotic therapy evaluated by determining the viable count recoverable from abscesses after treatment. Previous

studies with this model have indicated the need for adequate concentrations of ciprofloxacin at the site of infection in order to inhibit bacteria and prevent the development of resistance (Sulavik and Barg, 1998; Doss *et al.*, 1995). This model has fewer procedural difficulties than an invasive model such as the prosthetic implant model of Cagni *et al* (1995) or the endocarditis model of Entenza *et al* (1999). A neutropoenic thigh model has also been used to study the efficacy of quinolones, including moxifloxacin, *in vivo* (Dalhoff, 1999). This is similar to the subcutaneous abscess model, but takes into account the influence of immune status. Since *S. aureus* is the most common cause of nosocomial wound infections, the subcutaneous abscess model is considered to be particularly relevant to human infection (Bunce *et al.* 1992). By investigating one or more of these models it should be possible to predict the clinical progression of infection, resistance development, and potentially toxic side-effects in humans that cannot be determined from *in vitro* data alone.

1.13 Aims of This Thesis

- To investigate the mechanisms of action of moxifloxacin, a new 8-methoxyquinolone, against standard laboratory strains and clinical isolates of *S. aureus*.

- To investigate the development of quinolone resistance in *S. aureus* conferred by mutations in DNA gyrase, topoisomerase IV and an efflux system, and determine the primary and secondary molecular targets of moxifloxacin.

- To investigate the bactericidal activity of moxifloxacin in a murine staphylococcal abscess model, and make a comparison between the development of resistance *in vivo* and *in vitro*.

- To investigate the efficacy of moxifloxacin against quinolone-resistant strains of *S. aureus* with identified mutations, and determine the effect of these mutations on the mechanisms of action.

Chapter 2: Materials and Methods

2.1 Strains

Bacterial strains used in this project are listed in Table 2.1. All isolates were stored at -70°C in nutrient broth containing 10% glycerol and were subcultured onto Mueller Hinton agar (Oxoid, Basingstoke, UK) plates before use.

Table 2.1 : Standard and clinical strains used in this study.

Bacterial Strain	Characteristics	Source
S. aureus NCTC 6571	Laboratory Standard	
S. aureus NCTC 8325/4	Laboratory Standard	Dr C. Gemmel
S. aureus ED3	Clinical isolate	Royal Infirmary of Edinburgh diagnostic laboratories.
S. aureus ED5	Clinical isolate	
S. aureus ED7	Clinical isolate	
S. aureus ED9	Clinical isolate	
S. aureus E3T	Laboratory Standard	Dr I. Morrissey
E. coli NCTC 10418	Laboratory Standard	
P. aeruginosa NCTC 662	Laboratory Standard	

2.2 Media

All media powders were obtained from Oxoid (Basingstoke, UK) and made up with distilled water according to the manufacturer's instructions. For double strength media twice the given weight of powder was added to a single volume of water.

Prior to use all media were sterilised by autoclaving at 121°C and 15psi for 15 minutes to destroy all vegetative cells and spores.

Media used in this study were Mueller Hinton agar (MHA), Nutrient Broth No. 2 (NB) and Brain Heart Infusion broth (BHI).

2.3 Materials

2.3.1 Antimicrobial Agents

Antimicrobial agents, manufacturers and solubility information are listed in Table 2.2. Fresh stock solutions of all antibiotics were prepared on the day of use.

Table 2.2 : Antimicrobial agents and their solvents

Antimicrobial	Supplier	Solvent
Moxifloxacin	Bayer AG, Wuppertal, Germany	Water
Ciprofloxacin	Bayer plc, Newbury, UK.	Water
Trovafloxacin	Pfizer, Sandwich, UK	0.1M NaOH* and water
Grepafloxacin	Glaxo Wellcome, UK	Water
Sparfloxacin	Dainippon, Japan	0.1M NaOH* and water
Tetracycline	Sigma Chemicals, Poole, UK	Water
Chloramphenicol	Sigma Chemicals, Poole, UK	70% alcohol* and water

*Enough to dissolve powder

2.3.2 Chemicals and Buffers

All chemicals were purchased from Sigma Chemicals (Poole, UK) unless otherwise stated and solutions made up with sterile distilled water. Saline was made up with 0.85% w/v NaCl and sterilised before use. Buffers were made according to the Data for Biochemical Research (1974).

2.4 In Vitro Methods

2.4.1 Susceptibility Testing

Susceptibility testing to determine the minimum inhibitory concentrations (MICs) of antibiotics was performed by the agar dilution method (British Society for Antimicrobial Chemotherapy Working Party, 1991). Isolates were grown overnight in 10mL of BHI at 37°C in a shaking incubator. Bacterial cultures were then diluted to 10^{-4} in sterile saline and inoculated onto MHA plates containing serial doubling dilutions of antibiotic with a multipoint inoculator (Denley, UK). Plates were incubated overnight at 37°C. The MIC was defined as the lowest concentration of antibiotic to fully inhibit bacterial growth. *E. coli* NCTC 10418, *P. aeruginosa* NCTC 662, *S. aureus* NCTC 6571, *S. aureus* E3T and *S. aureus* ED3 were used as control strains.

2.4.2 Narrow Range MICs and the Effect of Reserpine

To determine precise concentrations of ciprofloxacin or moxifloxacin required for mutant selection plates, further sensitivity testing was performed with modification of the method described in section 2.4.1 as follows. A range of antibiotic concentrations increasing by small increments from just below to just above the MIC were investigated instead of doubling dilutions. In some experiments plates also contained 10 or 20mg/L of reserpine in addition to antibiotic in order to determine the effect of selective inhibition of efflux.

2.4.3 Viable Counts

Bacterial strains grown overnight in BHI were subcultured to log phase by adding 0.5mL to 9.5mL of BHI and incubating at 37°C with shaking for two hours. Serial dilutions of 1 in 10 or 1 in 100 were made in sterile saline. This was achieved by adding 1mL of culture to 9.9mL or 0.1mL of culture to 9.9mL of saline. Aliquots of 0.1mL from each dilution were evenly spread onto MHA plates with a sterile glass spreader. Plates were allowed to dry on the bench for 30 minutes and then incubated overnight at 37°C after which time the number of colony forming units (cfus) per plate was determined with a colony counter (Anderman, Kingston-upon-Thames, UK). The viable count in the undiluted culture was estimated by taking the dilution factor into consideration.

2.4.4 Optimum Bactericidal Concentration

In order to determine the optimum bactericidal concentration (OBC) of moxifloxacin, dilutions of a stock solution of antibiotic made up to 4.8mL with distilled water were added to 5mL aliquots of double strength BHI broth, to give final concentrations in 10mLs of 0.01, 0.1, 1.0, 10 and 100mg/L. All tubes were warmed to 37°C for 15 minutes.

Bacteria were grown overnight in BHI, then subcultured to log phase and 0.2mL added to each warmed tube containing moxifloxacin. After incubation for 3 hours at 37°C viable counts were determined as described in section 2.4.3.

2.4.5 Time-kill Kinetics

Bacteria were grown overnight in 10mL BHI broth and cultured to log phase in two tubes as described in 2.4.3. One tube was centrifuged at 3000 rpm for 20 minutes, the supernatant decanted, and the cell pellet resuspended in 10mLs sterile phosphate buffered saline (PBS).

Three tubes were set up as follows. All contained sufficient moxifloxacin to give a final concentration of 1mg/L in 10mLs. Two tubes had 5mL of double strength BHI broth added to them, and chloramphenicol was also added to one of these tubes to give a concentration of 20mg/L in 10mLs. In the third tube double strength PBS was substituted for BHI broth. The volume in each tube was made up to 9.8mLs with sterile distilled water. Tubes were warmed to 37°C for 15 minutes before 0.2mL of

log phase culture was added to each. All tubes were incubated for 3.5 hours at 37°C and viable counts determined as described in section 2.4.3.

2.4.6 Selecting Quinolone-resistant Mutants

Bacteria were grown overnight in BHI and subcultured to late log phase by adding 0.5mL of overnight culture to 9.5mL of BHI and incubating at 37°C with shaking for two hours. Quinolone-resistant mutants were selected by spreading 0.1mL of log phase bacterial culture ($\leq 1 \times 10^{10}$ cfus) onto MHA plates containing moxifloxacin or ciprofloxacin. The concentrations increased in small increments from the MIC upwards, instead of multiples of the MIC suggested by Ferrero *et al.*, (1995). All plates were incubated for 72 hours at 37°C and the number of mutants per plate recorded. Mutants were subcultured on MHA plates to check for purity prior to storage at -70°C in 10% glycerol. Viable counts were performed simultaneously with mutant selection so that mutation frequencies could be determined.

2.4.7 Genomic DNA Extraction

Genomic DNA was extracted from bacterial cells by a rapid extraction method (Ünal *et al.* 1997). Bacteria were cultured overnight in 10mL of BHI broth and cells from 0.2mL aliquots pelleted by microfugation at 1300rpm for 30 seconds. Each cell pellet was resuspended in 50µL of 100mg/L lysostaphin and incubated at 37°C for 10 minutes. Following incubation 50µL of 100mg/L proteinase K, and 150µL of 0.1M Tris buffer (pH 7.5) were added. The DNA extract mixture was incubated for a

further 10 minutes at 37°C, followed by 5 minutes in a water bath at 100°C. Samples were cooled on ice until required and 10μL aliquots used in each PCR reaction mixture.

2.4.8 Amplification of QRDRs by the Polymerase Chain Reaction

The quinolone resistance determining regions of *gyrA*, *gyrB* and *grlA* were amplified by the polymerase chain reaction (PCR). The primers used for each PCR are shown in Table 2.3 below. Primers for *gyrA* and *grlA* were designed using Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer) based on published gene sequences (Ferrero *et al.* 1994; Margerrison and Hopewell, 1992) and synthesized by Immunogen International Ltd (Sunderland, UK). The primers used for amplification of the *gyrB* fragment had previously been described by Takahashi *et al* (1998).

Table 2.3 : PCR primers

Gene (Accession no.)	PCR Primers	Sequence Reference
gyrA (M86227)	5′ -GACTTCTAAGCGCTGTGAAC 3′ -AAGTTACCTTGGCCATCAAC	(Margerrison and Hopewell, 1992)
grlA (L25288)	5′ -TGTTTTAGGTGATCGCTTTGG 3′ -GGCAATACCATTGGTTCGAG	(Ferrero *et al.* 1994)
gyrB	5′ -AAGTCGCACGTACAGTGGTT 3′ -CTGTACCAAATGCTGTGATC	(Takahashi *et al.* 1998)

PCR reactions were performed in a total volume of 100μL containing all reagents as shown in Table 2.4. A negative control consisting of the PCR reaction mixture without DNA template was included for each run to detect bacterial contamination. The content of each tube was overlaid with 50μL of mineral oil to prevent evaporation of PCR reaction components.

Table 2.4 : Components of PCR reaction mixture

Component	Stock Concentration	Final Concentration	Volume (μL)
5′ Primer	10ρmol/μL	10ρmol	1
3′ Primer	10ρmol/μL	10ρmol	1
dNTPs*	4mM	200μM	5
Tris-HCl/KCl**	200mM Tris/ 500mM KCl	20mM Tris/ 50mM KCl	10
$MgCl_2$	50mM	2.5mM	5
milliQ H_2O	-	-	66
DNA extract	-	-	10
Taq polymerase	1 Unit/μL	2 Units	2

* Boehringer Mannheim, Lewes, E. Sussex

** Promega, Southampton

Thermal cycling was performed on a Techne PHC-2 Dri-Block Cycler (Cambridge Bioscience, Cambridge) with an initial denaturation step of 94°C for 30 seconds,

55°C for 30 seconds and 72°C for 10 minutes. This was followed by 30 cycles of 1 minute at 90°C for denaturation, 2 minutes at 42°C for annealing and 3 minutes at 72°C for polymerisation. The final extension step was 5 minutes at 72°C.

2.4.9 Rapid Typing by Repetitive Extragenic Palindromic-PCR

Rapid typing by repetitive extragenic palindromic-PCR (Rep-PCR) was performed on crude DNA extracts obtained using the method described in section 2.4.7. Each reaction mixture contained 10μL of template DNA. PCR primers REP1R-Dt and REP2-Dt (Versalovic *et al.* 1993; Versalovic *et al.* 1991) were used at a concentration of 50pmol, with dNTPs at a concentration of 1.25mM in a total reaction mixture volume of 100μL. All other components of the PCR reaction were used at the concentrations shown in Table 2.4. PCR was performed employing the cycling parameters described by Versalovic *et al.*, (1991) beginning with an initial denaturation step of 95°C for 7 minutes. This was followed by 30 cycles of denaturation at 90°C for 30 seconds, annealing at 40°C for 1 minute and extension at 65°C for 8 minutes. A single final extension step of 65°C for 16 minutes concluded the cycling program.

2.4.10 Visualisation of PCR Products by Agarose Gel Electrophoresis

PCR products were visualised by electrophoresis of samples on a 2% w/v agarose gel. Electrophoresis grade agarose (Gibco BRL, Life Technologies, Paisley, UK) was dissolved in 40mM Tris-acetic acid/2mM EDTA, pH 8.0 (TAE) buffer.

Ethidium bromide stock was added to the gel giving a final concentration of 0.5mg/L. Electrophoresis was carried out in a tank containing TAE as running buffer. Each 10μL sample of PCR product was mixed with 2μL of loading buffer consisting of 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, and 30% w/v sucrose. A standard molecular weight marker - λ DNA cut with *Hin*dIII or 100bp ladder (Gibco BRL) - was also loaded in one lane on every gel. Samples were electrophoresed at 20mA (100V) until the migrating loading buffer fronts had moved two thirds of the way down the gel. After electrophoresis, banding patterns could be observed and recorded by placing the gel on a TM 40E UV transilluminator (UV Products, Cambridge; UV wavelength 302nm) and photographing with a Polaroid camera fitted with an orange filter.

2.4.11 Purification and Quantification of PCR Products

PCR products were purified using a QIAquick PCR purification kit (QUIAGEN Ltd, Crawley, UK) according to the manufacturer's instructions. The purified PCR products were eluted from the column into 30μL of elution buffer instead of 100μL in order to concentrate the DNA. The approximate amount of DNA per 30μL sample was quantified after electrophoresis on a 2% w/v agarose gel by comparing the brightness of the sample bands with neat and diluted samples of pUC19 (Gibco BRL), a standard marker known to have 250ng of DNA per microlitre.

2.4.12 Automatic DNA Sequencing

The primers used for PCR amplification were also used for DNA sequencing, with 3.2pmol of primer required per sequencing sample. The DNA content of each sample to be sequenced was determined as described in section 2.4.11 to ensure that it contained 30-90ng of DNA. An ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Warrington, UK) was used following the manufacturer's instructions and sequencing cycles were performed on a Techne Cyclogene Dri-Block Thermal Cycler (Cambridge Biosciences). The cycle sequencing program consisting of 25 repetitions of 96°C for 30 seconds, 45°C for 15 seconds and 60°C for 4 minutes. Extension products were purified by the rapid ethanol precipitation method suggested by the sequencing kit manufacturer. Automatic DNA sequencing was determined by Edman degradation performed on an Applied Biosystems Procise Sequencer. A sample densitometry trace showing a typical sequence obtained from automatic sequencing of the QRDR is given in Appendix VII. All results were compared to the published sequences detailed in Table 2.3, by the BLAST online search facility (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast?Jform=0) and differences in sequences noted.

2.4.13 Radio-labelled Moxifloxacin Uptake Assay

The uptake of [^{14}C]-moxifloxacin was assayed by an established method (Kaatz and Seo, 1995; Kaatz *et al.* 1993). Bacterial strains were grown overnight in BHI and then subcultured to log phase in 20mL of BHI. Cells were concentrated by centrifuging at 3000rpm at 4°C for 20 minutes. The supernatant was removed and

cell pellets were resuspended in 1mL of BHI. Aliquots of 790µL were dispensed into sterile eppendorf tubes and diluted 1 in 5 by adding 200µL of fresh BHI. All samples were stored on ice until required.

A stock solution was prepared containing 100mg/L of [^{14}C]-moxifloxacin with an activity of 5.88kBq. Each 0.990mL cell suspension was exposed to 10µL of this stock giving a final moxifloxacin challenge concentration of 1mg/L (2.94kBq). All suspensions were placed in a water bath at 37°C for the duration of the assay. Aliquots of 40µL were removed at timed intervals of 0, 1.5, 3, 4.5, 6, 10, 15, 20, 25 and 30 minutes after the radio-labelled moxifloxacin was added. In some experiments carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at a final concentration of 100µM was added after 15 minutes to inhibit the proton motive force.

Each 40µL sample was filtered through a 13mm, 0.45µm GN-6 Metricel Membrane disc filter (Pall Gelman Sciences) and then washed through with 6mL of sterile saline. Filters were allowed to dry and then placed in scintillation vials (Canberra Packard, Zurich, Switzerland) to which 1mL of scintillation fluid (Canberra Packard) was added. The activity of each sample, given as counts per minute, was determined by a scintillation counter (Canberra Packard).

2.4.14 Radio-labelled Moxifloxacin Efflux Assay

The efflux of [^{14}C]-moxifloxacin from bacterial cells was determined by a washing assay as follows. Strains were cultured to log phase and concentrated 20-fold as described in section 2.4.13. Each sample was challenged with moxifloxacin as described previously, and then incubated at 37°C for 10 minutes. Aliquots were removed from each test sample for filtration as previously described, and then each sample was washed and resuspended in fresh BHI. The washing step was repeated at least 6 times for each strain, and 40μL samples were removed and filtered at every step. Simultaneously, 10μL aliquots were also removed at each washing step so that the viable count could be determined (see section 2.4.3). All filters were dried and the activity determined by scintillation counting as before.

2.5 In Vivo Methods

2.5.1 Induction of Subcutaneous Abscesses in a Mouse Model

The *in vivo* efficacy of moxifloxacin against *S. aureus* was investigated using a murine subcutaneous abscess model (Bunce *et al.* 1992). Eight week old male C3H mice were divided into cohorts of 10 animals. Each cohort was allocated a specific ear marking with all animals within the cohort receiving the same mark. The flanks of all animals were shaved and the weights of all animals were noted.

Bacteria were cultured overnight in 10mL of BHI broth and then grown to log phase and an optical density (590nm) of 0.2, equivalent to 10^8 cfu/mL as previously

determined from a standard laboratory curve. A 500μL culture sample was mixed with 310μL of BHI broth and 190μL of Cytodex-1 microcarrier beads. Each mouse was injected subcutaneously on the right flank with 200μL of this mixture with a 1mL syringe (Beckton-Dickinson UK Ltd, Cowley, Oxford) and a 25-gauge syringe. Care was taken to ensure that the bevel of the needle faced upwards when the injection was made to ensure localised inoculation and consistency of injections. All animals were left for 48 hours to allow abscesses to develop fully.

2.5.2 Establishing Moxifloxacin Concentrations in Serum and Abscesses

In order to establish the concentrations of moxifloxacin achievable in subcutaneous abscesses, two cohorts of mice were infected with *S. aureus* NCTC 8325/4 as described previously. After 48 hours, all the animals were injected with 200μL of a range of moxifloxacin concentrations (dissolved in sterile PBS) on the left flank and 30 minutes later ocular blood samples were taken. The animals were sacrificed after three hours and the abscesses excised. All abscess material was frozen in PBS and sent together with the blood samples to Caroline Tobin (Southmead Hospital, Bristol) for determination of moxifloxacin concentration by high performance liquid chromatography (HPLC) analysis. Plotting the concentration detected by HPLC versus the dose concentration given to the animal produced a standard curve which could be used to determine the correct dose required to produce specific concentrations within an abscess.

2.5.3 Determining Bacterial Survival from Subcutaneous Abscesses

Subcutaneous staphylococcal abscesses were established in four cohorts of mice as described in section 2.5.1. Three cohorts were treated with 200μL of moxifloxacin (dissolved in sterile PBS) at a concentration determined from a standard curve to reach 1, 2 or 4 times the MIC of NCTC 8325/4 within the abscesses. The mice were dosed every two hours for 24 hours, with all 10 animals within a cohort receiving the same dose of moxifloxacin. As a control a fourth cohort was treated in the same manner with 200μL of PBS alone. The animals were sacrificed an hour after the final injection, and the abscesses excised and placed into PBS. All of the abscesses were homogenised and the homogenate supplemented with PBS to give a total volume of 1mL. The homogenate was diluted in sterile saline and viable counts determined as described previously (section 2.4.3).

2.5.4 Selection of *In vivo* Mutants

The possibility of moxifloxacin selecting for quinolone-resistant mutants *in vivo* was investigated by inoculating agar plates containing moxifloxacin with 0.1mL of neat abscess homogenate as described in section 2.4.6. The number of mutants per plate was counted after 72 hours of incubation at 37°C and mutation frequencies were determined based on the viable count. Mutants were subcultured for purity and stored at –70°C in 10% glycerol. The Rep-PCR typing method described in section 2.4.9 was employed to confirm that mutants recovered were not contaminants.

Chapter 3: Results – Mechanisms of Action

Historically the quinolones have been used to target Gram-negative bacteria and have had poor activity against Gram-positive bacteria (Smith and Zeiler, 1998). Moxifloxacin is a new 8-methoxyquinolone with enhanced activity against Gram-positive organisms compared to older agents such as ciprofloxacin (Alcala *et al.* 1999; Boswell *et al.* 1999; Al-Nawas and Shah, 1998; Bauernfeind, 1997; Dalhoff *et al.* 1996). By determining the OBC and examining the time-kill kinetics of this drug against standard laboratory strains and clinical isolates of *S. aureus,* an assessment of *in vitro* efficacy can be made.

3.1 Susceptibility Testing

Three well characterised standard laboratory strains and four clinical strains of *S. aureus* were chosen for this project. Clinical strains ED3 and ED5 had previously been identified as ciprofloxacin and methicillin resistant, while strains ED7 and ED9 were ciprofloxacin and methicillin sensitive. The MICs of all strains are shown in Table 3.1. All standard *S. aureus* strains were sensitive to moxifloxacin, ciprofloxacin and chloramphenicol. The *E. coli* and *P. aeruginosa* control strains were also sensitive to ciprofloxacin, but were resistant to chloramphenicol. *P. aeruginosa* was resistant to moxifloxacin, with an MIC of 1.0mg/L.

Clinical isolates ED3 and ED5 were resistant to moxifloxacin and to ciprofloxacin compared to the control strains. Isolates ED7 and ED9 were sensitive to both moxifloxacin and ciprofloxacin, although ED9 was slightly less sensitive to ciprofloxacin than ED7 (MIC of 0.12mg/L compared to 0.03mg/L). All clinical isolates were sensitive to chloramphenicol.

Table 3.1: MICs of 9 strains used in this study

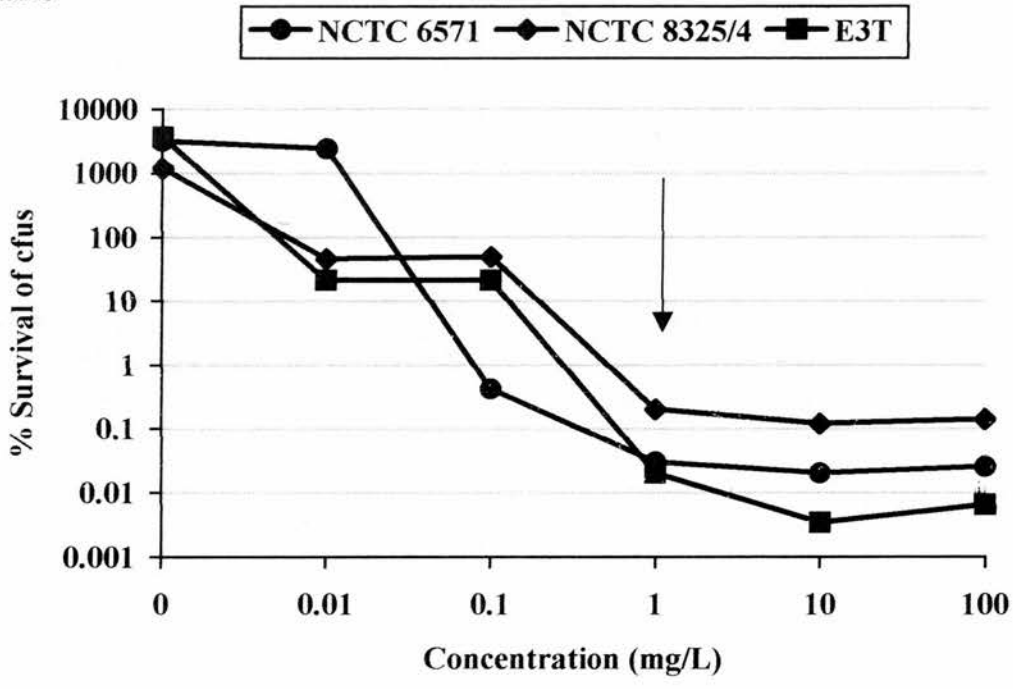
	MIC in (mg/L)		
Strain	Moxifloxacin	Ciprofloxacin	Chloramphenicol
S. aureus NCTC 6571	0.03	0.03	2
S. aureus NCTC 8325/4	0.06	0.50	2
S. aureus ED3	2.00	>8.00	4
S. aureus ED5	2.00	>8.00	4
S. aureus ED7	0.06	0.03	4
S. aureus ED9	0.06	0.12	4
S. aureus E3T	0.03	0.03	2
E. coli NCTC 10418	0.03	<0.03	>32
P. aeruginosa NCTC 662	1.00	0.12	>32

3.2 The Optimum Bactericidal Concentration of Moxifloxacin

The OBC is the minimum concentration of quinolone giving the maximum killing effect, above which killing decreases due to inhibition of RNA synthesis (Smith, 1984). The OBC of moxifloxacin was determined for laboratory standards NCTC 6571, 8325/4 and E3T, by challenging log phase cultures with increasing concentrations of moxifloxacin for three hours. Figure 3.1 shows the three dose

response curves of these strains when challenged with moxifloxacin. Concentration-dependent killing was exhibited by moxifloxacin against all strains, however the classic biphasic killing curve described by Smith (1984) was not observed. This means that there is not a true 'optimum' bactericidal concentration of moxifloxacin, although a concentration more accurately described as the 'maximum' bactericidal concentration is easily observed. This concentration of moxifloxacin for all strains was approximately 1.0mg/L, as indicated by an arrow on the figure.

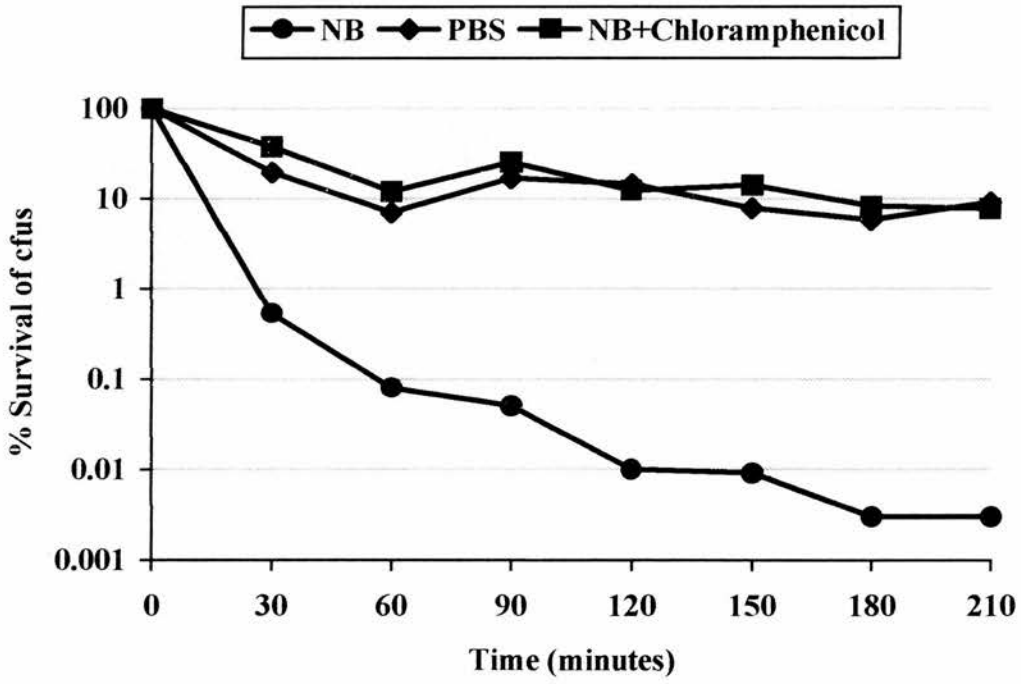
Figure 3.1: Determining the OBC of moxifloxacin against standard laboratory strains



3.3 Mechanisms of Moxifloxacin Action Against S. aureus

Having established that the maximum killing activity of moxifloxacin is achieved at 1.0mg/L against standard laboratory strains of *S. aureus*, this concentration was used to challenge cultures under different growth conditions in order to investigate the presence of different mechanisms of quinolone action (Smith, 1984). Bacteria were subjected to moxifloxacin challenge in broth with or without the protein synthesis inhibitor chloramphenicol, or in PBS. Samples were removed at 30 minute intervals and the number of surviving cfus determined.

Figure 3.2: Time-kill kinetics of 1mg/L of moxifloxacin against *S. aureus* NCTC 6571 in NB, PBS, or NB containing 20mg/L chloramphenicol



The results for standard strain NCTC 6571 are shown in Figure 3.2. Moxifloxacin showed good bactericidal activity against this strain at 1.0mg/L with less than 1%

survival by 30 minutes after challenge. Inhibition of protein synthesis by the addition of a bacteriostatic concentration of chloramphenicol did not completely remove the killing activity of moxifloxacin. Less than 10% survival was observed after 180 minutes when chloramphenicol was included. Similarly, inhibiting cell division by challenging the strain in PBS rather than nutrient broth did not completely remove the killing activity. Less than 10% survival was observed 150 minutes after challenge. Similar results were observed with strains NCTC 8325/4 and E3T as shown in Figures 3.3 and 3.4. Moxifloxacin showed good bactericidal activity against both strains at 1.0mg/L, and also retained activity against both strains even if protein synthesis and cell division were inhibited.

Figure 3.3: Time-kill kinetics of 1mg/L of moxifloxacin against *S. aureus* NCTC 8325/4 in NB, PBS, or NB containing 20mg/L chloramphenicol

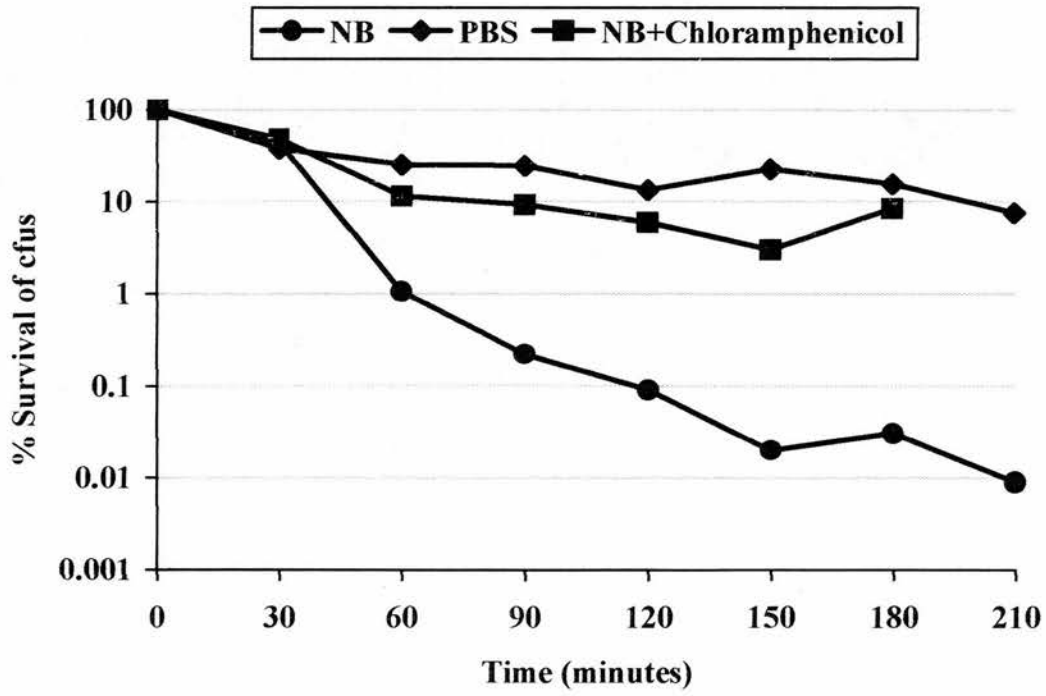
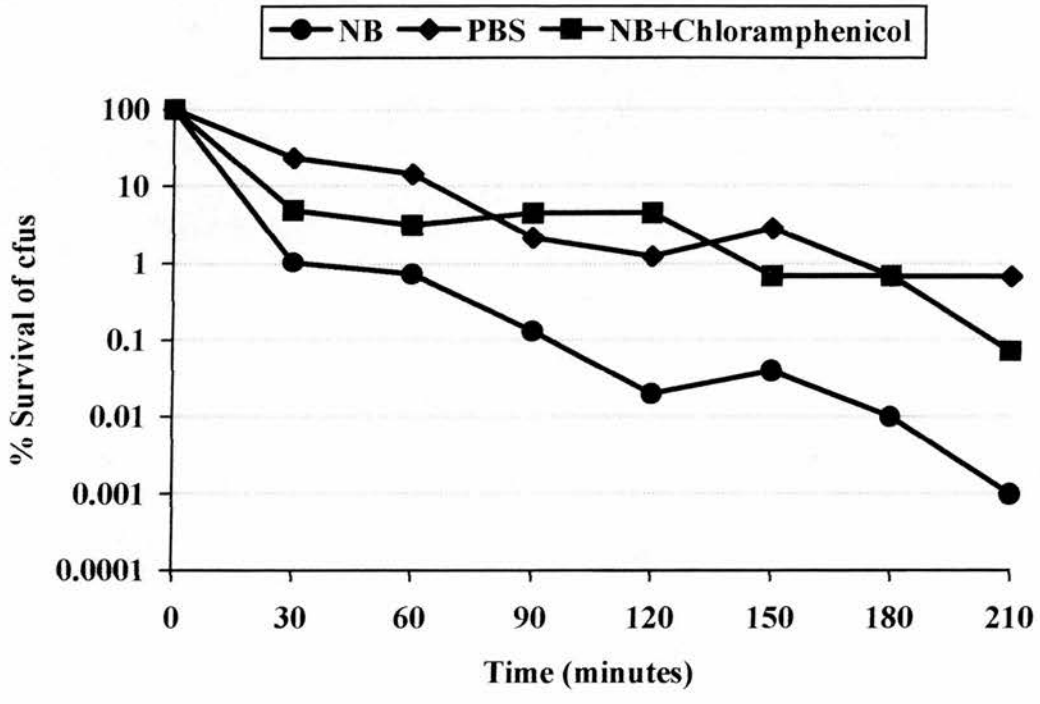


Figure 3.4: Time-kill kinetics of 1mg/L of moxifloxacin against S. aureus E3T in NB, PBS, or NB containing 20mg/L chloramphenicol



3.4 Activity of Moxifloxacin Against Clinical S. aureus Isolates

Although the mechanisms of action may be easy to demonstrate against standard sensitive laboratory strains, it is not clear whether the same results will be obtained if clinical isolates are challenged instead. Moxifloxacin at 1.0mg/L was used to challenge four clinical isolates of *S. aureus*. Log phase cultures of each strain were exposed to this concentration for 210 minutes at 37°C, and the number of viable colonies determined at 30 minute intervals.

3.4.1 Clinical Strains ED3 and ED5

Since 1.0mg/L is equivalent to only half the MIC for strains ED3 and ED5, this concentration was not bactericidal. These strains were therefore also challenged with 4, 8 and 16mg/L (2, 4 and 8 times MIC). The results for strain ED3, which is both quinolone and methicillin resistant, are shown in Figure 3.5.

Moxifloxacin is not bactericidal against this strain at 1.0mg/L - in fact the bacterial population is able to multiply. However, good bactericidal activity is restored at 4mg/L and higher concentrations, with survival decreasing to less than 1% between 60 and 90 minutes after challenge. A similar result was observed with strain ED5, which is also quinolone and methicillin resistant.

Figure 3.5: Clinical isolate ED3 challenged with 1, 4, 8 or 16mg/L moxifloxacin

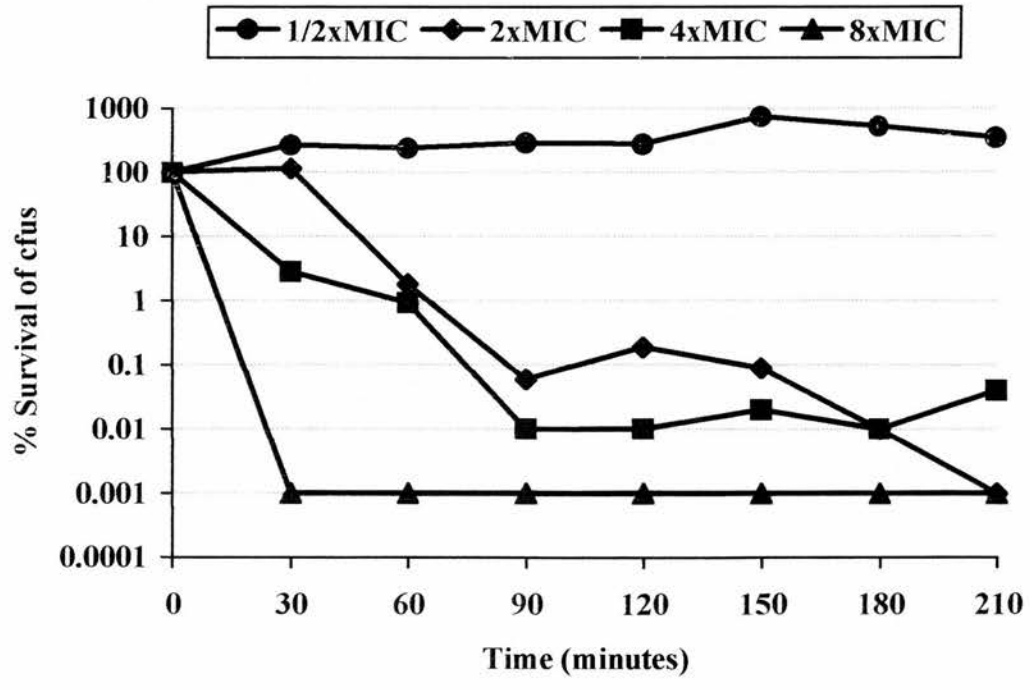
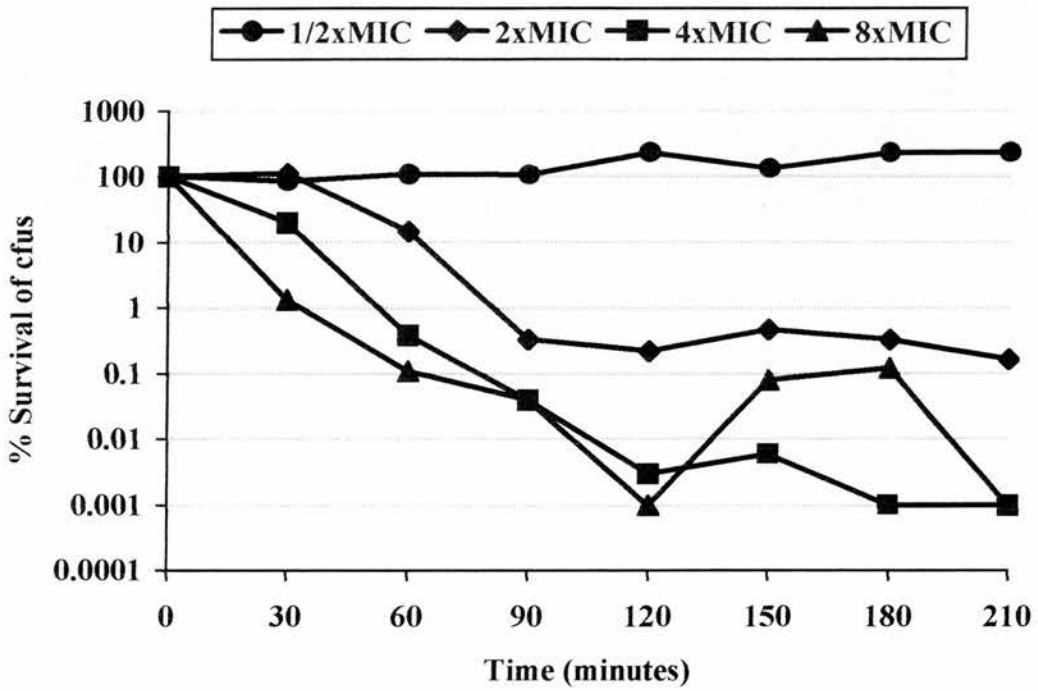


Figure 3.6: Clinical isolate ED5 challenged with 1, 4, 8 or 16mg/L moxifloxacin

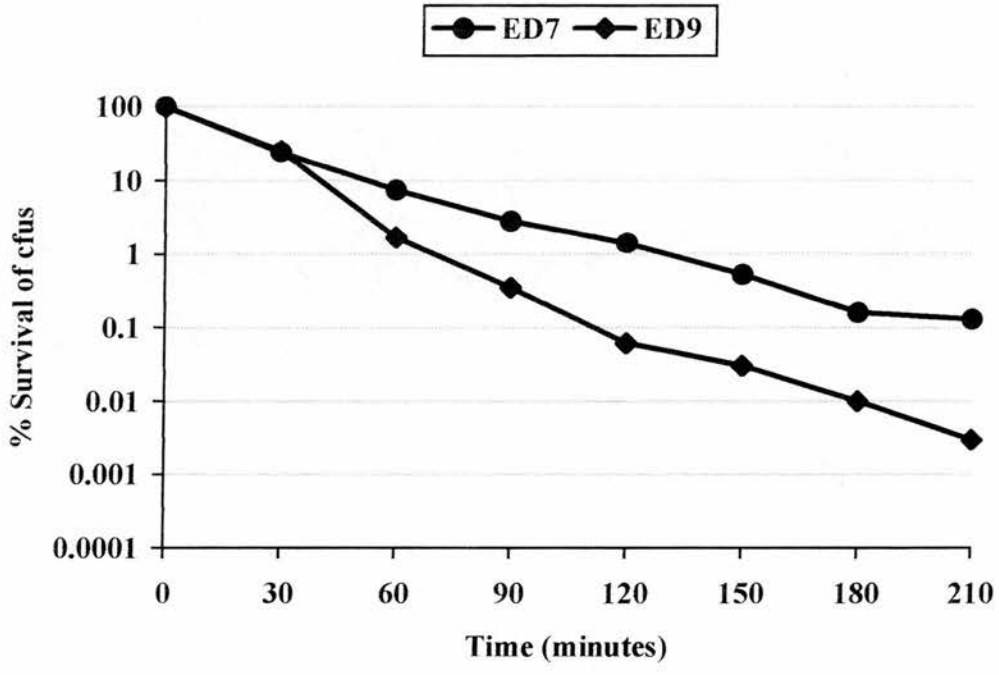


The results in Figure 3.6 show that 1.0mg/L is not bactericidal against ED5, although killing is restored at 4.0mg/L (2 times MIC) with less than 1% survival after 90 minutes.

3.4.2 Clinical Strains ED7 and ED9

Two other clinical strains were challenged with 1.0mg/L of moxifloxacin. Strains ED7 and ED9 were quinolone and methicillin sensitive. Figure 3.7 shows the effect of this concentration on both strains. Moxifloxacin showed significant bactericidal activity at 1.0mg/L against both of these strains although the rate of killing was

Figure 3.7: Clinical isolates ED7 and ED9 challenged with 1mg/L moxifloxacin



slower than previously observed against standard laboratory strains (Figures 3.2 - 3.4). Survival dropped to below 1% between 60 and 90 minutes after challenge of strain ED7, and between 120 and 150 minutes after challenge of strain ED9.

3.5 Time-kill Kinetics of Moxifloxacin Against Clinical Strains

The mechanisms of action of moxifloxacin against these four clinical strains of *S. aureus* were investigated at 1.0mg/L to determine whether the different resistance profiles had any effect on the mechanisms of action.

3.5.1 Mechanisms of Action Against ED3 and ED5

Resistant clinical isolates ED3 and ED5 were challenged with 1.0mg/L of moxifloxacin in nutrient broth with or without chloramphenicol, or in PBS in the same manner as standard laboratory strains. The results are shown in Figures 3.8 and 3.9.

No killing activity is observed against either strain in nutrient broth alone. The addition of chloramphenicol does not have any effect on the killing rate either, with similar growth rates observed with and without this inhibitor. These results may be predicted by the fact that neither of these strains is sensitive to moxifloxacin at 1.0mg/L, which is only half the MIC.

Figure 3.8: Time-kill kinetics of 1mg/L of moxifloxacin against ED3 in NB, PBS, or NB containing 20mg/L chloramphenicol

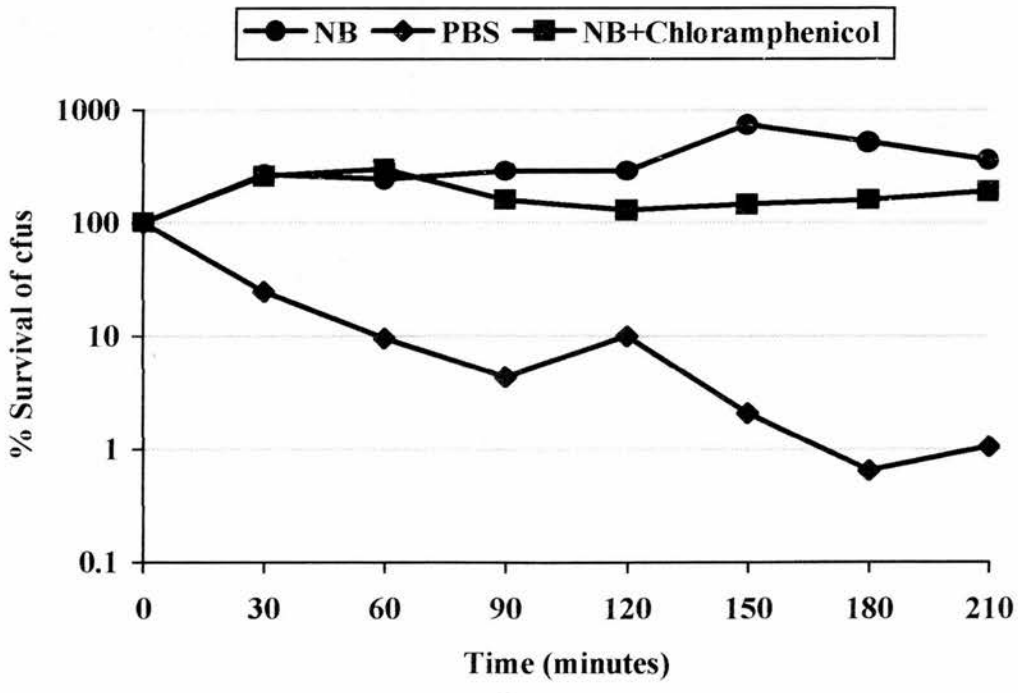
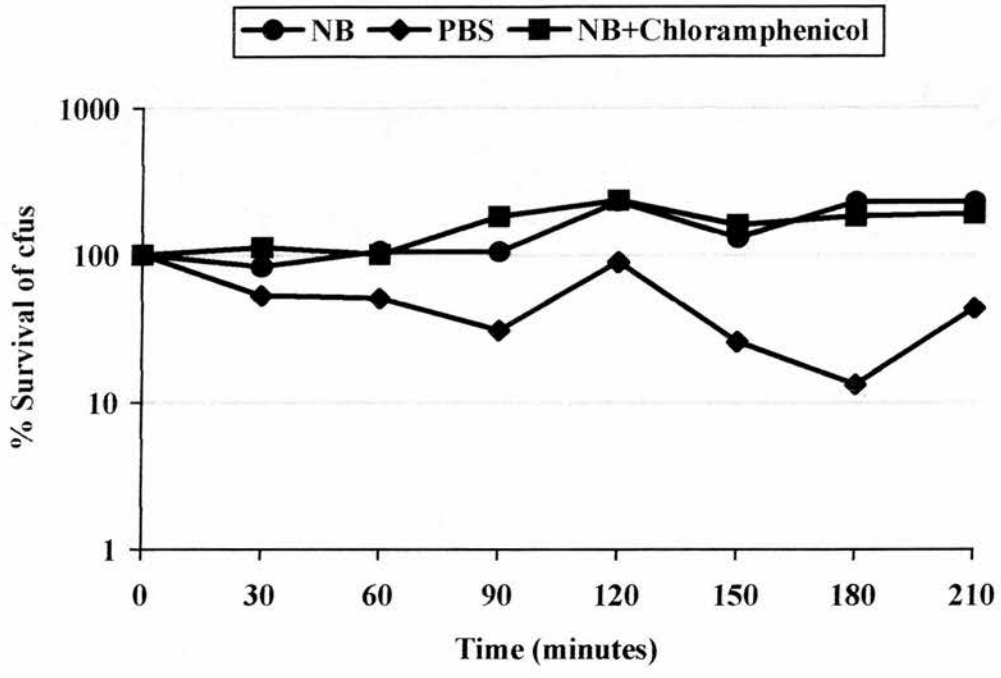


Figure 3.9: Time-kill kinetics of 1mg/L of moxifloxacin against ED5 in NB, PBS, or NB containing 20mg/L chloramphenicol



Although both strains have less sensitivity to chloramphenicol than the standard laboratory strains, this difference is not sufficient to have an effect since the bacteriostatic concentration used was 20mg/L.

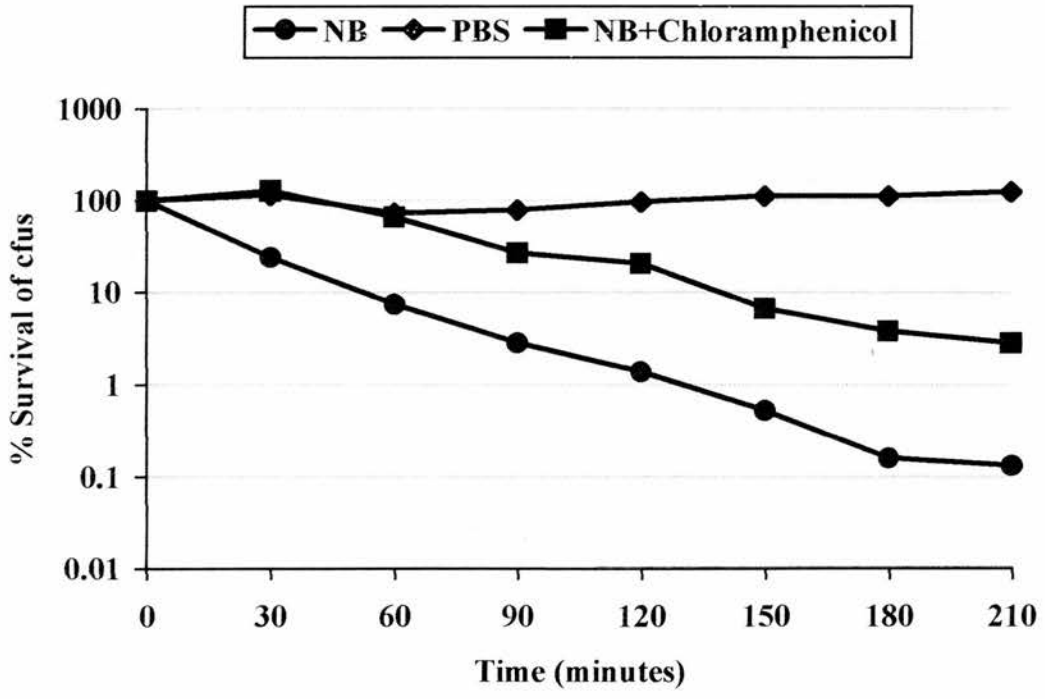
However, the presence or absence of active cell division seems to have some influence on the quinolone killing of these resistant strains. Both ED3 and ED5 were killed by moxifloxacin at 1.0mg/L when exposed in PBS rather than nutrient broth. Survival of strain ED3 decreased to approximately 1% after 210 minutes, while ED5 was better able to survive with approximately 40% of cfus surviving to 210 minutes.

3.5.2 Mechanisms of Action Against ED7 and ED9

The mechanisms of action against clinical isolates ED7 and ED9, were investigated. Unlike sensitive laboratory strains that are specifically chosen as standards because of their sensitive characteristics, these clinical strains may carry random mutations in a wide variety of genes. Studying such strains isolated from infected patients may give a more realistic representation of the efficacy that moxifloxacin is likely to have against clinical pathogens, compared to standard laboratory strains which may not be representative of pathogenic strains.

Figure 3.10 shows the time-kill kinetics obtained by challenging strain ED7 with 1.0mg/L of moxifloxacin. As with the standard laboratory strains, ED7 is quickly

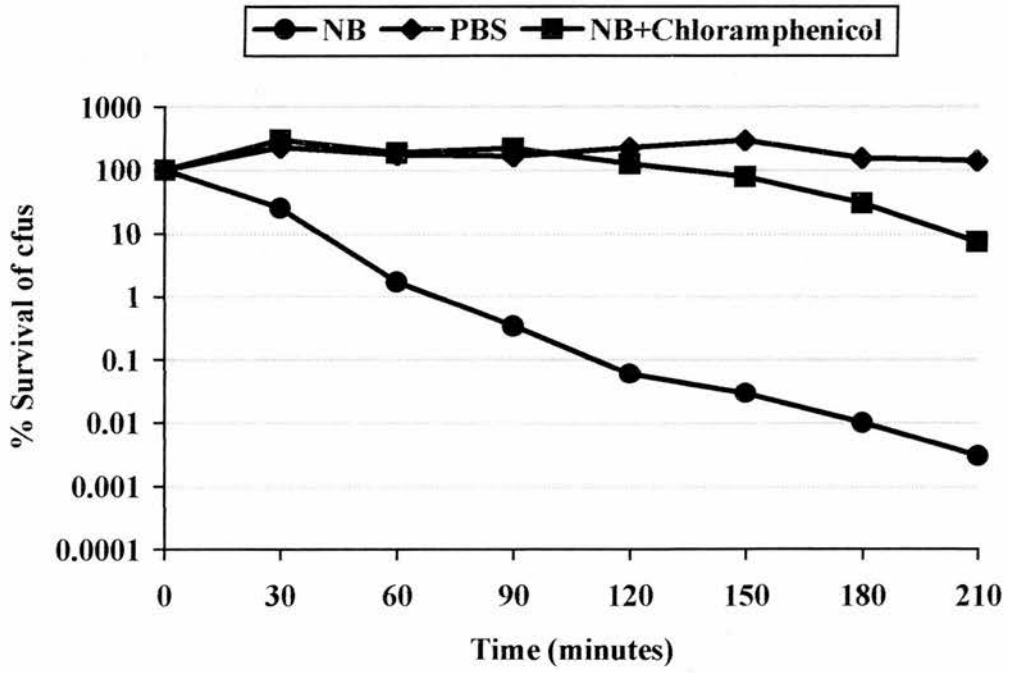
Figure 3.10: Time-kill kinetics of 1mg/L of moxifloxacin against ED7 in NB, PBS, or NB containing 20mg/L chloramphenicol



killed by 1.0mg/L of moxifloxacin in nutrient broth, and up to 97% of cfus are killed if a bacteriostatic concentration of chloramphenicol is also present. However, suspending the culture in PBS to prevent cell division prior to challenge with quinolone seems to negate any killing mechanisms otherwise present. When exposed to moxifloxacin in PBS no killing occurred. This seems to be the opposite result to those obtained with strains ED3 and ED5.

The results for strain ED9, shown in Figure 3.11, are very similar to those obtained for ED7. This strain is killed in nutrient broth alone with less than 1% survival by 90 minutes, and is also killed in the presence of chloramphenicol with less than 10% of cfus surviving by 210 minutes. However, no killing is observed when the ED9 was incubated with moxifloxacin in PBS.

Figure 3.11: Time-kill kinetics of 1mg/L of moxifloxacin against ED9 in NB, PBS, or NB containing 20mg/L chloramphenicol



Chapter 4: Results – Mutations in DNA gyrase and Topoisomerase IV

Quinolones interact with the transient cleavable complexes formed between DNA gyrase or topoisomerase IV, and DNA in an undetermined manner, resulting in lethal breaks in the DNA strands (Maxwell and Critchlow, 1998). Resistance to the quinolones can arise through a variety of point mutations at hotspots located primarily within the QRDRs of *gyrA* or *grlA* (Everett and Piddock, 1998). Studies of the development of these mutations have indicated that DNA gyrase is the primary target of quinolones in Gram-negative organisms. In contrast, topoisomerase IV is the proposed primary target in Gram-positive organisms, based primarily on work with ciprofloxacin (Ferrero *et al.* 1995). Ciprofloxacin has good activity against Gram-negative organisms but relatively poor activity against Gram-positive organisms. Characterisation of the QRDRs of sequentially selected mutants of moxifloxacin provides an opportunity to investigate the hypothesis that new anti-Gram-positive quinolones may cause different mutational changes and have different molecular targets to older agents.

4.1 Mutation Frequencies and Sensitivity to Moxifloxacin

Moxifloxacin-resistant mutants of *S. aureus* strain NCTC 8325/4 were selected by spreading 100μL of culture onto plates containing concentrations of moxifloxacin

increasing by small increments above the MIC of the parent. The viable count of mutants per plate on plates with less than 50 cfus was determined at each concentration, and a viable count was performed in parallel on non-selective plates so that mutation frequencies could be determined.

4.1.1 First-step Moxifloxacin-Selected Mutants

Following the mutant selection process, randomly chosen strains were isolated from each plate. Each strain was subcultured onto a fresh MHA plate to establish purity, then designated with a culture collection number and stored at –70°C prior to sensitivity testing. All strains were subcultured from storage and the MICs of moxifloxacin determined by agar dilution using a narrow range of concentrations.

The MICs of 86 first-step mutants, their fold increases in resistance and the concentrations at which they were selected are shown in Appendix I. The parent strain had an MIC of 0.05mg/L and resistant mutants could be isolated on plates containing 0.125, 0.150, 0.175 and 0.200mg/L of moxifloxacin, but all growth was inhibited at higher concentrations. The majority of strains tested showed decreased sensitivity to moxifloxacin, with MICs between 1.5 and 5 fold higher than the parent strain. Strains were most commonly found to have an MIC of 0.100 or 0.125 mg/L (27 and 37 strains respectively), equivalent to a 2 or 2.5 fold decrease in sensitivity compared to strain NCTC 8325/4. A small number of strains were found to have MICs with an increase of only 1.5 fold or less. These strains were not considered to be true resistant mutants.

Eleven strains with moxifloxacin MICs of 0.125mg/L were chosen at random for further study. These strains, highlighted in grey in Appendix I, are shown in Table 4.1 along with the parent strain NCTC 8325/4. Eight of these strains were selected as first-step parent strains from which second generation mutants were selected. These parent strains are shown below the double line in Table 4.1.

Table 4.1: First-step moxifloxacin-selected mutants chosen for further study

Isolate Number	Selection Concentration (mg/L)	MIC (mg/L)
NCTC 8325/4*	-	0.05
ED170	0.125	0.125
ED209	0.150	0.125
ED216	0.175	0.125
ED223	0.200	0.125
ED229	0.200	0.125
ED231	0.200	0.125
ED233	0.200	0.125
ED235	0.200	0.125
ED237	0.200	0.125
ED239	0.200	0.125
ED240	0.200	0.125

* Parent Strain

4.1.2 Second-step Moxifloxacin-Selected Mutants

The MICs of 49 second-step mutants, their parent strains, increases in resistance and the concentrations at which they were selected are shown in Appendix II. Resistant

strains could be isolated on plates containing up to 0.8mg/L of moxifloxacin. All the strains tested showed decreased sensitivity to moxifloxacin, with MICs increasing by 4 to 40 fold compared to the primary parent NCTC 8325/4 (1.5 to 16 fold higher than their first-step parent strains). Strains were most commonly found to have an MIC of 0.8mg/L (22 strains), equivalent to a 16 fold increase in MIC compared to strain NCTC 8325/4. Four strains with MICs of 0.8mg/L, were selected as parent strains for the next generation of mutants. These strains (highlighted in grey in Appendix II) are shown in Table 4.2.

Table 4.2: Second-step moxifloxacin-selected mutants chosen for further study

Parent Strain	Isolate Number	Selection Concentration (mg/L)	MIC (mg/L)
ED223	ED261	0.4	0.8
ED229	ED266	0.4	0.8
	ED267	0.4	0.8
	ED268	0.4	0.8

4.1.3 Third-step Moxifloxacin-Selected Mutants

The MICs of 62 third-step mutants, their parent strains, increases in resistance and the concentrations at which they were selected are shown in Appendix III. Resistant strains could be isolated on plates containing up to 4.2mg/L of moxifloxacin. All the strains tested showed decreased sensitivity to moxifloxacin, with MICs increasing by 20 to 90 fold compared to the primary parent NCTC 8325/4 (1.3 to 5.6 fold higher than their second-step parent strains). Strains were most commonly found to have an

MIC of 4.25 – 4.50mg/L (25 strains), equivalent to an MIC 85 or 90 times higher than the primary parent strain NCTC 8325/4. Five strains with MICs of 4.25 – 4.5mg/L, were selected for further investigation. These strains are highlighted in grey in Appendix III. Viable counts and mutation frequencies of first-, second- and third-step mutants were determined. The mutation frequency was calculated with the formula mf = n / vca, where mf is the mutation frequency, n is the number of mutants on a plate and vca is the viable count of cfus per 100μL aliquot determined from the non-selective plates. Table 4.3 shows the mean viable counts, mean mutant counts and mutation frequencies of each mutant selection step.

Table 4.3: Mean mutation frequencies of all parent strains

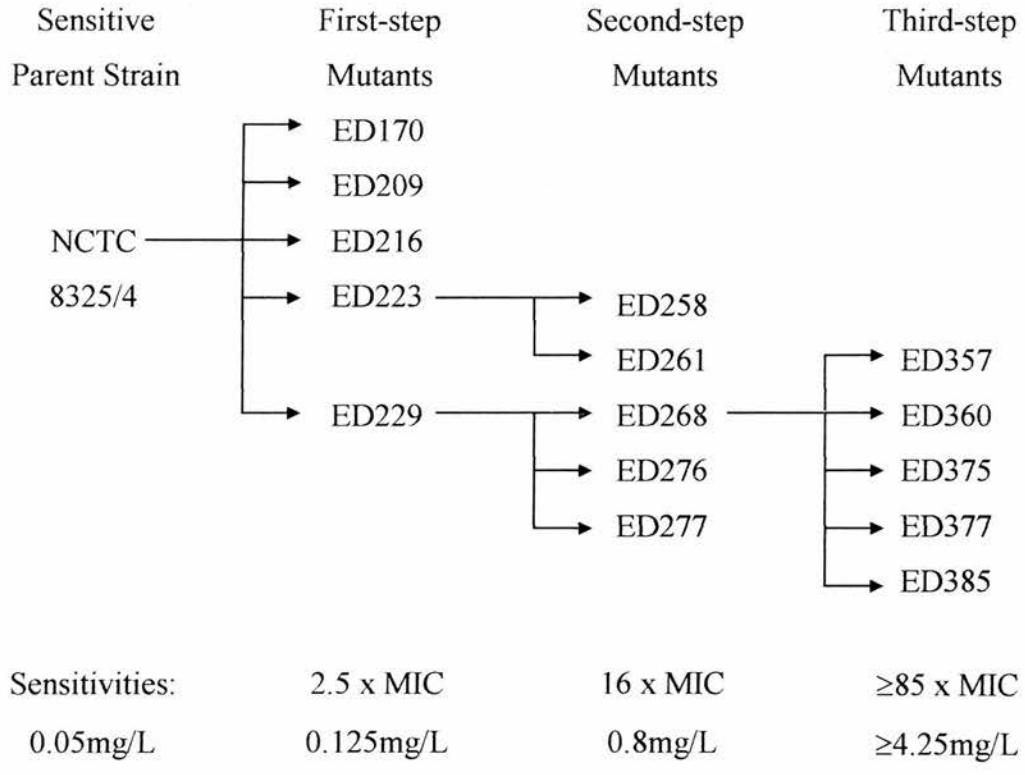
Parent Strain	Mutation Step	Mean Viable Count/100μL	Mean Mutant Count/100μL	Mutation Frequency
NCTC 8325/4	1st Step	9.3×10^{7}	21.5	2.3×10^{-7}
ED223	2nd Step	6.78×10^{10}	2.67	3.9×10^{-11}
ED229	2nd Step	3.96×10^{10}	3.33	8.4×10^{-11}
ED268	3rd Step	5.9×10^{10}	4.83	8.2×10^{-11}

It can been seen from the table that mutants are selected less readily with increasing mutant generations i.e. as strains become more resistant it is harder to select still higher levels of resistance. However, the mean viable counts were surprisingly high for the second and third mutation steps (around 10^{10} compared to 10^{7} for the first mutation step), and this may explain why the mutation frequencies seemed to be unusually low for these strains.

4.2 Characterisation of Moxifloxacin-Selected Mutant Strains

Five strains from each mutant generation were chosen for further investigation by PCR amplification and DNA sequencing of the QRDRs of the *gyrA* and *grlA* genes. After each mutation step it was clear that mutants with specific levels of resistance were more commonly selected. Strains for further investigation were therefore chosen from these modal groups.

Figure 4.1: Relationship between characterised mutants



First-step mutants with an MIC of moxifloxacin of 0.125mg/L, second-step mutants with an MIC of 0.8mg/L and third-step mutants with an MIC of 4.25 – 4.5mg/L were

chosen. Figure 4.1 gives a schematic representation of the relationship between the 16 chosen strains.

4.2.1 Sensitivities of Moxifloxacin-Selected Mutants to Other Quinolones

The sensitivities of strain NCTC 8325/4 and the 15 resistant strains to three other quinolones were established. MICs were determined by the standard doubling dilution method (see 2.4.1). Ciprofloxacin is an older quinolone with good activity against Gram-negative organisms, while sparfloxacin and trovafloxacin are newer agents developed primarily to target Gram-positive organisms. The sensitivities of all 16 strains to these three agents as well as moxifloxacin are shown in Table 4.4. The MICs increased with each consecutive generation of mutants for all four quinolones tested. Interestingly, although all five mutants within each generation were found to have the same MIC of moxifloxacin, this was not always the case with the other agents tested. From Table 4.4 it can be seen that ciprofloxacin MICs varied by one dilution for first-step mutants, and two dilutions for second-step mutants (MIC increase of 2 – 4 fold higher than NCTC 8325/4). The breakpoint sensitivity concentration of ciprofloxacin for *S. aureus*, above which organisms are classed as clinically resistant, is 2mg/L (British Society for Antimicrobial Chemotherapy Working Party, 1991). Clinically significant levels of resistance were therefore determined in two out of five second-step mutants (ED258 and ED261) that had MICs of 8mg/L, and all third-step mutants had high levels of resistance with MICs 32mg/L.

Table 4.4: MICs of four quinolones for moxifloxacin-selected resistant strains

S. aureus strain	MICs in mg/L			
	Ciprofloxacin	**Sparfloxacin**	**Trovafloxacin**	**Moxifloxacin**
NCTC 8325/4	0.5	0.06	0.06	0.06
First-step				
ED170	2	0.25	0.12	0.12
ED209	1	0.06	0.06	0.12
ED216	2	0.06	0.12	0.12
ED223	2	0.25	0.25	0.12
ED229	1	0.06	0.12	0.12
Second-step				
ED258	8	1	1	1
ED261	8	1	1	1
ED268	2	1	0.25	1
ED276	2	1	0.25	1
ED277	2	1	0.5	1
Third-step				
ED357	32	16	4	8
ED360	32	16	4	8
ED375	32	16	4	8
ED377	32	16	4	8
ED385	32	16	4	8

First-step mutants were more sensitive to sparfloxacin than to ciprofloxacin or moxifloxacin (Table 4.4), with only two strains (ED170 and ED223) showing increases in MIC (4 fold). Both strains with increased MICs of sparfloxacin also had

increased MICs of ciprofloxacin. Strains ED209, ED216 and ED229 were as sensitive to sparfloxacin as the parent strain. All second-step and third-step mutants were equally resistant to sparfloxacin (MICs of 1.0 and 16mg/L respectively). Although a breakpoint value is not yet available for sparfloxacin, all third generation strains have sufficiently high MICs that their resistance levels may be considered clinically relevant.

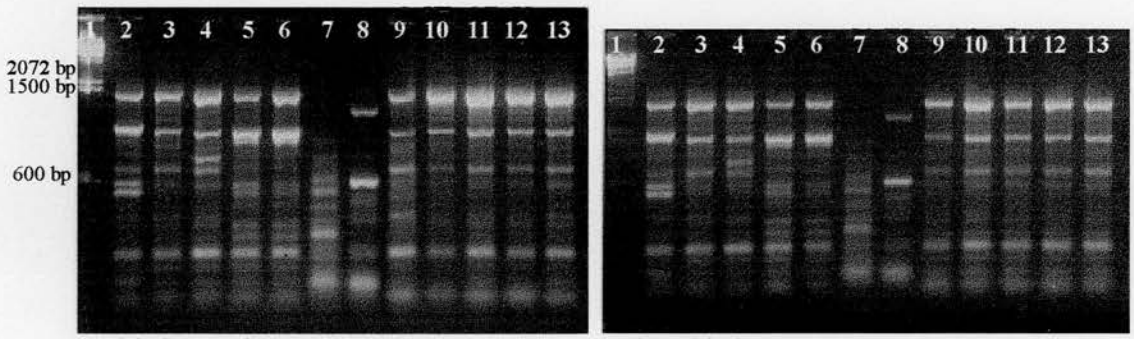
Overall, trovafloxacin MICs were lower compared to the other three quinolones (Table 4.4). Similar to sparfloxacin, strains ED170 and ED223 showed an increase in resistance (2 and 4 fold respectively). The other three first-step mutants were fully sensitive to trovafloxacin. The MICs of second-step mutants correlated closely with ciprofloxacin MICs: strains ED258 and ED261 had higher MICs (1.0mg/L) than the other three strains. The MIC of all third-step mutants was 4.0mg/L, constituting a smaller change in sensitivity from primary parent to third generation mutants than was observed for any of the other quinolones. Although breakpoints are currently unavailable for trovafloxacin, these third-step strains are likely to be classified as clinically resistant.

4.2.2 Strain Typing by Rep-PCR

In order to ascertain that all mutant strains were directly related to the parent strain NCTC 8325/4 and to exclude the possibility of environmental contamination, the strains were examined using a rapid PCR-based typing method (section 2.4.9) and compared to a number of control organisms. In Figure 4.2 the control organisms

S. aureus NCTC 6571, NCTC 8325/4, E3T, ED3, ED5, *E. coli* NCTC 104018 and *P. aeruginosa* NCTC 662 are shown in lanes 2 to 8 on each gel. Lane 1 contains the standard 100bp ladder marker. Distinct banding patterns are observed for each organism and strain. The banding patterns for ED3 and ED5 appear to be the same, indicating that they may be clones with the same origin (i.e. from the same patient).

Figure 4.2: Rapid typing patterns generated by Rep-PCR



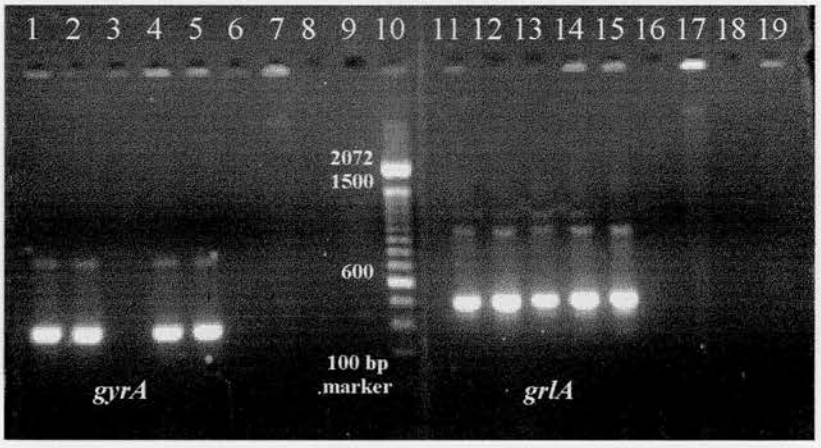
(a) Second-step Mutants (b) Third-step Mutants

The gels in Figure 4.2 show the Rep-PCR patterns for second-step mutants (a) and third-step mutants (b) in lanes 9 to 13. It is clear from the banding patterns of the control strains that this typing method is able to discriminate between different strains of *S. aureus* as well as different organisms. The parent strain *S. aureus* NCTC 8325/4 has a distinct banding pattern from all other strains tested. The mutant strains have identical banding patterns to parent strain NCTC 8325/4 and to each other. All first-step mutants also had the same banding pattern.

4.2.3 QRDR Analysis

The DNA extraction and PCR protocols described in section 2.4 were tested with five *S. aureus* strains and four negative controls to verify amplification of the correct size of DNA fragment. Fragment sizes of 374 bases for *gyrA* and 434 bases for *grlA* gene were confirmed against a standard 100 base pair ladder. The results are shown in Figure 4.3. The brightest bands of the 100 base pair ladder correspond to fragments of 2072, 1500 and 600 bases (read top to bottom). PCR products amplified with *gyrA* primers are to the left of the 100 base pair ladder (lane 10), and products amplified with *grlA* primers are shown to the right.

Figure 4.3: Confirmation of *gyrA* and *grlA* fragment sizes



Lanes 1 to 5 show the *gyrA* PCR products from *S. aureus* strains NCTC 6571, NCTC 8325/4, E3T, ED3 and ED5 respectively. Bands of the expected size (between 300 and 400 bases) were observed for all strains except for E3T. It is possible that no PCR product was obtained for strain E3T because the DNA extraction process was unsuccessful, or perhaps resulted from a missing component in the PCR reaction mixture. Subsequent repeat PCR reactions with this strain yielded a fragment of the

expect size. Where a second band of approximately twice the size of the desired product is also visible, this is likely to be a dimer of PCR product due to overloading of the gel. No bands were observed for the negative controls *E. coli* NCTC 10418 (lane 6) and *P. aeruginosa* NCTC 662 (lane 7). Two further negative controls were included to screen PCR reagents for possible bacterial contamination as follows: PCR reaction mixture without DNA extract (lane 8); PCR reaction mixture with DNA extract (strain NCTC 6571) but no primers (lane 9). The *grlA* PCR products of all five *S. aureus* strains were loaded in lanes 11 to 15 in the same order as before. Bands of the expected size (400 to 500 bases) were observed for all strains. No bands were observed for any of the negative controls (lanes 16 to 19).

4.2.4 PCR Amplification and Sequencing of the QRDRs of GyrA and GrlA

After establishing that the appropriate size of gene fragments could be amplified with the chosen PCR primers, the QRDRs of *gyrA* and *grlA* for all 16 strains, detailed in Table 4.4, were amplified and purified as described in section 2.4.11. The QRDR of each gene was amplified and sequenced twice from two different DNA extracts for each strain. The approximate DNA content of each purified PCR product was quantified to between 30 and 90ng/μL and submitted for automatic DNA sequencing. The nucleotide and amino acid sequences of the QRDR of wild-type *S. aureus gyrA* and *grlA* genes are shown in Figures 4.4 and 4.5. The key mutation hotspots are at codons 84, 85 and 88 in *gyrA* and 80 and 84 in *grlA*, although mutations have also been found outwith these codons. These codons (shaded in grey in the figures) encode serine and glutamic acid in a wild-type strain.

Figure 4.4: QRDR fragment of wild-type *S. aureus gyrA* gene

```
  ACAATTTATAGAAGATAATGCAGTTTATGCAAACTTA GAC TTC TAA GCG CTG      2252
    Q  F  I  E  D  N  A  V  Y  A  N  L   D   F  ***

TGA ACT GAA CTT TTG AAG GAG GAA CTC TTG ATG GCT GAA TTA CCT      2297
                                       ------->
                                        M   A   E   L   P

CAA TCA AGA ATA AAT GAA CGA AAT ATT ACC AGT GAA ATG CGT GAA      2342
 Q   S   R   I   N   E   R   N   I   T   S   E   M   R   E

TCA TTT TTA GAT TAT GCG ATG AGT GTT ATC GTT GCT CGT GCA TTG      2387
 S   F   L   D   Y   A   M   S   V   I   V   A   R   A   L

CCA GAT GTT CGT GAC GGT TTA AAA CCA GTA CAT CGT CGT ATA CTA      2432
 P   D   V   R   D   G   L   K   P   V   H   R   R   I   L

TAT GGA TTA AAT GAA CAA GGT ATG ACA CCG GAT AAA TCA TAT AAA      2477
 Y   G   L   N   E   Q   G   M   T   P   D   K   S   Y   K

AAA TCA GCA CGT ATC GTT GGT GAC GTA ATG GGT AAA TAT CAC CCT      2522
 K   S   A   R   I   V   G   D   V   M   G   K   Y   H   P

CAT GGT GAC TCA TCT ATT TAT GAA GCA ATG GTA CGT ATG GCT CAA      2567
 H   G   D   S   S   I   Y   E   A   M   V   R   M   A   Q

GAT TTC AGT TAT CGT TAT CCG CTT GTT GAT GGC CAA GGT AAC TTT      2612
 D   F   S   Y   R   Y   P   L   V   D   G   Q   G   N   F

GGT TCA ATG GAT GGA GAT GGC GCA GCA GCA ATG ......              2646
 G   S   M   D   G   D   G   A   A   A   M ......
```

*** denotes stop codon; underlining indicates primer sequence locations

-------▶ indicates beginning of *gyrA* gene;

Figure 4.5: QRDR fragment of wild-type *S. aureus grlA* gene

```
  GTGAAATAATTCAAGATTTATCACTTGAA GAT GTT TTA GGT GAT CGC TTT      2085
     E  I  I  Q  D  L  S  L  E   D  V  L  G  D  R  F

GGA AGA TAT AGT AAA TAT ATT ATT CAA GAG CGT GCA TTG CCA GAT      2130
 G  R  Y  S  K  Y  I  I  Q  E  R  A  L  P  D
                          -------▶

GTT CGT GAT GGT TTA AAA CCA GTA CAA CGT CGT ATT TTA TAC GCA      2175
 V  R  D  G  L  K  P  V  Q  R  R  I  L  Y  A

ATG TAT TCA AGT GGT AAT ACA CAC GAT AAA AAT TTC CGT AAA AAT      2220
 M  Y  S  S  G  N  T  H  D  K  N  F  R  K  N

TTC CGT AAA AGT GCG AAA ACA GTC GGT GAT GTT ATT GGT CAA TAT      2265
 F  R  K  S  A  K  T  V  G  D  V  I  G  Q  Y

CAT CCA CAT GGA GAC TCC TCA GTG TAC GAA GCA ATG GTC CGT TTA      2310
 H  P  H  G  D  S  S  V  Y  E  A  M  V  R  L

AGT CAA GAC TGG AAG TTA CGA CAT GTC TTA ATA GAA ATG CAT GGT      2355
 S  Q  D  W  K  L  R  H  V  L  I  E  M  H  G

AAT AAT GGT AGT ATC GAT AAT GAT CCG CCA GCG GCA ATG CGT TAC      2400
 N  N  G  S  I  D  N  D  P  P  A  A  M  R  Y

ACT GAA GCT AAG TTA AGC TTA CTA GCT GAA GAG TTA TTA CGT GAT      2445
 T  E  A  K  L  S  L  L  A  E  E  L  L  R  D

ATT AAT AAA GAG ACA GTT TCT TTC ATT CCA AAC TAT GAT GAT ACG      2490
 I  N  K  E  T  V  S  F  I  P  N  Y  D  D  T

ACA CTC GAA CCA ATG GTA TTG CCA TCA AGA TTT ......               2535
 T  L  E  P  M  V  L  P  S  R  F
```

*** denotes stop codon;

-------▶ indicates beginning of *grlA* gene;

underlining indicates primer sequence locations

The DNA sequences of NCTC 8325/4 and all 15 mutant strains were compared to these wild-type sequences. Table 4.5 shows all mutations detected in these strains. All parent strains are indicated with grey shading. All first-step strains were selected from strain NCTC 8325/4. Second-step strains ED258 and ED261 were selected from ED223, while ED268, ED276 and ED277 were selected from ED229. All third-step strains were selected from ED268.

Table 4.5: Mutations in the QRDRs of *gyrA* and *grlA* of moxifloxacin-selected mutants.

	***gyrA* Mutations**			***grlA* Mutations**		
Strain	**Codon**	**Base**	**Amino Acid**	**Codon**	**Base**	**Amino Acid**
Parent						
8325/4		-			-	
1st Step						
ED170		-			-	
ED209		-			-	
ED216		-			-	
ED223		-		80	TCC→TTC	Ser→Phe
ED229		-			-	
2nd Step						
ED258	84	TCA→GCA	Ser→Ala	80	TCC→TTC	Ser→Phe
ED261	84	TCA→GCA	Ser→Ala	80	TCC→TTC	Ser→Phe
ED268	84	TCA→TTA	Ser→Leu		-	
ED276	84	TCA→TTA	Ser→Leu		-	
ED277	88	GAA→AAA	Glu→Lys		-	
3rd Step						
ED357	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
ED360	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
ED375	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
ED377	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
ED385	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys

A small decrease in quinolone sensitivity could be detected in first-step mutants by determination of MICs. However, a comparison of the *gyrA* QRDR sequences with parent strain NCTC 8325/4 did not reveal any nucleotide point mutations in mutant strains (Table 4.5). Strain ED223 had a point mutation at serine 80 altering the amino acid to phenylalanine, however this did not confer a higher level of resistance than was observed in the other four strains in which no mutations were detected. Clearly some other mutation outwith the QRDR, or another mechanism of resistance is responsible for the decreased quinolone sensitivity in strains ED170, ED209, ED216 and ED229.

Second-step mutant strains ED258 and ED261 were derived from first-step mutant strain ED223, and sequencing analysis revealed that both strains had inherited the *grlA* Ser80 to Phe mutation present in the parent strain (Table 4.5). All five second-step mutants had alterations at either Ser84 or Glu88 in *gyrA*, although strains ED268, ED276 and ED277 had no detectable mutation in *grlA*. All five strains had an associated large increase in MIC. The double mutation in strains ED258 and ED261 did not confer higher MICs of moxifloxacin or sparfloxacin on these strains. However, MICs of ciprofloxacin and trovafloxacin were one dilution higher for these strains than for ED268, ED276 and ED277. Although three different point mutations within two different codons (84 and 88) were detected in *gyrA*, all apparently conferred the same change in resistance to moxifloxacin.

All third-step mutant strains had the Ser84 to Leu mutation already detected in the parent strain ED268. In addition all five strains had the same Glu84 to Lys mutation in topoisomerase IV. All strains had high level resistance to the quinolones tested. The single *grlA* mutation identified in Table 4.5 was the only difference between these strains and their more sensitive second-step parent, therefore this mutation (or a combination of this mutation plus the *gyrA* mutation) is playing a significant part in the development from low- to high-level resistance.

4.3 Ciprofloxacin-selected Quinolone Resistant Mutants

The sequencing results obtained for mutants selected with moxifloxacin indicate that DNA gyrase could be the primary target of this quinolone rather than topoisomerase IV. This result could be due to the specific molecular structure of moxifloxacin that may allow it to have different interactions with the DNA gyrase-DNA complexes compared to other agents. In order to investigate the hypothesis that moxifloxacin and ciprofloxacin have different cellular targets, first- and second-step mutants were selected with ciprofloxacin by the method previously described for moxifloxacin (section 2.4.6).

4.3.1 Second-step Ciprofloxacin-selected Mutants

In the first instance, second-step mutants were selected from ED170, a first-step moxifloxacin-selected strain known to have no mutations within *gyrA* or *grlA*. This strain was assumed to have an efflux mutation that would account for the small

increase in moxifloxacin MIC. A first-step strain was chosen in order to circumvent any initial mutation occurring outwith the two target genes, and hence select progeny with a *gyrA* or *grlA* mutation in a single mutation step. Mutants selected with ciprofloxacin were screened in order to identify strains with moxifloxacin and ciprofloxacin MICs equivalent to, or higher than, those determined for second-step moxifloxacin-selected strains. Five strains were chosen for DNA sequencing. Table 4.6 details the MICs and mutations found to be present in these strains.

Table 4.6: Ciprofloxacin-selected second-step mutants

	MIC (mg/L)		Mutation		
Strain	**Cip**	**Moxi**	***gyrA***		***grlA***
ED565	>16	0.25	-	-	-
ED566	4	0.75	TCA→TTA	Ser84→Leu	-
ED567	8	0.25	-	-	-
ED568	16	0.5	TCA→GCA	Ser84→Ala	-
ED569	>16	0.75		-	-

Only two out of the five strains showed any mutation within the QRDRs of *gyrA* and *grlA*. Interestingly, the strains with the highest MICs of ciprofloxacin had no detectable mutations. None of these second-step mutants were found to have mutations in *grlA*. Strains ED566 and ED568 had a mutation at codon 84 in *gyrA*, and this result is the same as was found for moxifloxacin-selected second-step mutants (Table 4.5).

4.3.2 First-step Ciprofloxacin-selected Mutants

The second-step ciprofloxacin-selected mutants were progeny of a moxifloxacin-selected first-step parent strain. It is possible that undetermined effects of moxifloxacin on this parent strain could have influenced the outcome of mutant selection to second-step level with ciprofloxacin. To further test the hypothesis that ciprofloxacin targets topoisomerase IV and that therefore mutations arise in *grlA* before *gyrA*, first-step mutants were selected from the standard parent strain NCTC 8325/4 with ciprofloxacin by the method described previously (section 2.4.6).

Table 4.7: Ciprofloxacin-selected first-step mutants

	MIC (mg/L)		Mutation		
Strain	**Cip**	**Moxi**	***gyrA***	***grlA***	
ED571	2	0.25	-	-	-
ED572	2	0.12	-	TCC→TAC	Ser80→Tyr
ED573	2	0.25	-	-	-
ED574	1	0.06	-	-	-
ED575	2	0.12	-	-	-
ED576	1	0.25	-	-	-
ED577	1	0.25	-	-	-

Seven first-step mutants were selected for DNA sequencing. The results are shown in Table 4.7. The results were similar to those described previously for moxifloxacin (Table 4.5). No mutations were detected in *gyrA* in any of the strains, and although all strains had increased MICs of ciprofloxacin compared to parent strain NCTC 8325/4 only one strain (ED572) was found to have a mutation within *grlA*. The

mutation detected in strain ED572 was at codon 80 and did not elevate the MIC to a concentration greater than determined for the other six strains with unknown mutations.

Chapter 5: Results – Non-gyrA- or –grlA-mediated Resistance.

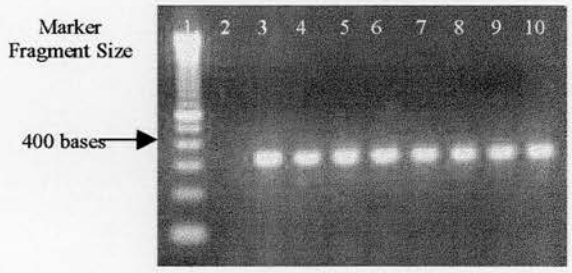
Quinolone resistance in *S. aureus* is not only mediated by changes within the A subunits of DNA gyrase and topoisomerase IV, but has also been attributed in part to mutations within *gyrB* or *grlB*, and enhanced efflux pump expression. However, it is believed that individually these mechanisms confer only low levels of resistance, and that they can only contribute to high level resistance in combination with *gyrA* and *grlA* mutations. The small decrease in sensitivity that they confer may be important in prolonging the survival of the bacterium after the initial quinolone challenge. This in turn may provide sufficient time to enable the bacterium to develop higher levels of resistance through *gyrA* or *grlA* mutations. Chapter 4 described a series of step-wise selected quinolone resistant mutants that were investigated for the presence of mutations in the QRDRs of *gyrA* or *grlA*. Several first-step mutants had no mutations within the QRDRs and were subjected to further investigation.

5.1 Mutations in GyrB

Of the five first-step moxifloxacin-selected mutants characterised for *gyrA* or *grlA* mutations by DNA sequencing, only ED223 was found to have a *grlA* mutation. The decreased quinolone sensitivity observed by sensitivity testing of the other four strains must therefore be due to a mutation or mutations elsewhere in the genome.

PCR amplification of the *gyrB* gene was performed using the cycle parameters and primers described by Takahashi *et al* (1998). A 327 base fragment of *gyrB* was amplified successfully for the parent strain and all five first-step mutants as shown in Figure 5.1 (Lanes 4 and 6 to 10 respectively). Lane 1 shows the 100 base pair ladder; Lane 2 shows *E. coli* NCTC 10418 as a negative control; Lanes 3 and 5 contain *S. aureus* NCTC 6571 and strain ED3 respectively as positive controls.

Figure 5.1: Amplified 327 base fragment of *gyrB*



The PCR products were purified, quantified and sequenced as previously described (sections 2.4.11 and 2.4.12). The resulting sequence data was compared to a wild-type *gyrB* gene (Margerrison and Hopewell, 1992), but no mutations could be detected within the amplified region.

5.2 Investigation of Efflux as a Resistance Mechanism

Enhanced efflux resulting from mutations in a promoter region, resulting in increased expression of the NorA multi-drug efflux pump of *S. aureus*, has been described as a mechanism of resistance to some quinolones. Over expression of this constitutive

pump may prolong the survival time of the bacterium after exposure to low concentrations of quinolone.

5.2.1 Sensitivity to Efflux Pump Substrates

A preliminary way of investigating the presence or absence of enhanced efflux pump expression is to examine the sensitivity profiles of bacteria to efflux pump substrates. Typical substrates include tetracycline, ethidium bromide and the quinolones, and MICs of these substrates may be determined by standard methods (British Society for Antimicrobial Chemotherapy Working Party, 1991). The addition of the plant alkaloid reserpine, a competitive efflux pump inhibitor, to the sensitivity testing medium often decreases the MIC of the substrate if a pump is being overexpressed.

The MICs of tetracycline, ethidium bromide and moxifloxacin (with and without reserpine) are shown in Table 5.1. The MIC of tetracycline was identical for all strains (0.25mg/L). The addition of reserpine at either 10 or 20mg/L did not have any effect on the MICs of moxifloxacin. Interestingly, the ethidium bromide MICs seem to be influenced by the presence of topoisomerase IV mutations. Strains ED223, ED258, ED261 and all third-step strains had an ethidium bromide MIC of 4mg/L compared to 2mg/L for all other strains, and these strains all had a mutation within the *grlA* QRDR. The MICs of moxifloxacin remained the same for all strains in the presence of 10mg/L or 20mg/L reserpine. Strain ED209 showed a one fold decrease in sensitivity to moxifloxacin, but this one fold difference is acceptable within the sensitivity limits of this experiment, and therefore is not significant.

Table 5.1: Sensitivity moxifloxacin-selected mutants to efflux pump substrates

	MICs in mg/L				
Strain	**Tetracycline**	**EthBr**	**Moxifloxacin**	**Moxifloxacin * + 10mg/L Reserpine**	**Moxifloxacin * + 20mg/L Reserpine**
8325/4	0.25	2	0.06	0.06	0.06
ED170	0.25	2	0.12	0.12	0.12
ED209	0.25	2	0.12	0.12	0.06
ED216	0.25	2	0.12	0.12	0.12
ED223	0.25	4	0.12	0.12	0.12
ED229	0.25	2	0.12	0.12	0.12
ED258	0.25	4	1	1	1
ED261	0.25	4	1	1	1
ED268	0.25	2	1	1	1
ED276	0.25	2	1	1	1
ED277	0.25	2	1	1	1
ED357	0.25	4	8	8	8
ED360	0.25	4	8	8	8
ED375	0.25	4	8	8	8
ED377	0.25	4	8	8	8
ED385	0.25	4	8	8	8

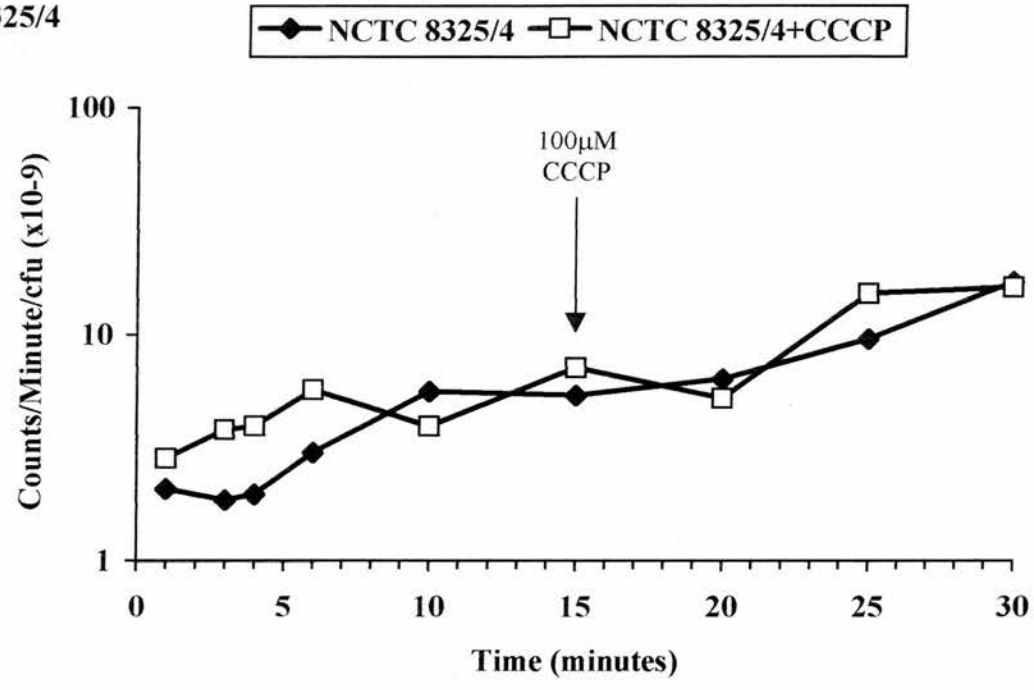
*Moxifloxacin at the concentrations shown in column 4

5.2.2 Assay for Efflux Activity in the Presence or Absence of CCCP

Several studies have shown the efficacy of efflux assays as an indication of the overexpression of efflux pumps in both *S. aureus* and *S. pneumoniae*. Efflux can be measured over time, in the presence or absence of the proton motive force inhibitor CCCP, by assaying the intracellular accumulation of radiolabelled quinolone.

Addition of the inhibitor prevents the active efflux process, facilitating build-up of radiolabelled drug within the cell.

Figure 5.2: Accumulation of radio-labelled moxifloxacin in *S. aureus* NCTC 8325/4



The accumulation of ^{14}C-moxifloxacin in the wild-type parent strain NCTC 8325/4 over a 30 minute period is shown in Figure 5.2. The concentration of detectable moxifloxacin increases gradually with time. When CCCP was added after 15 minutes, to inhibit the proton motive force and disable any efflux activity, a similar pattern of accumulation was detected. The patterns of moxifloxacin accumulation in first-step mutant strains ED170, ED209, ED216, ED223 and ED229 are shown in Figures 5.3 to 5.7. All of the accumulation patterns are similar to NCTC 8325/4, with none of the strains showing significant changes in accumulation with the addition of CCCP after 15 minutes.

Figure 5.3: Accumulation of radio-labelled moxifloxacin in strain ED170

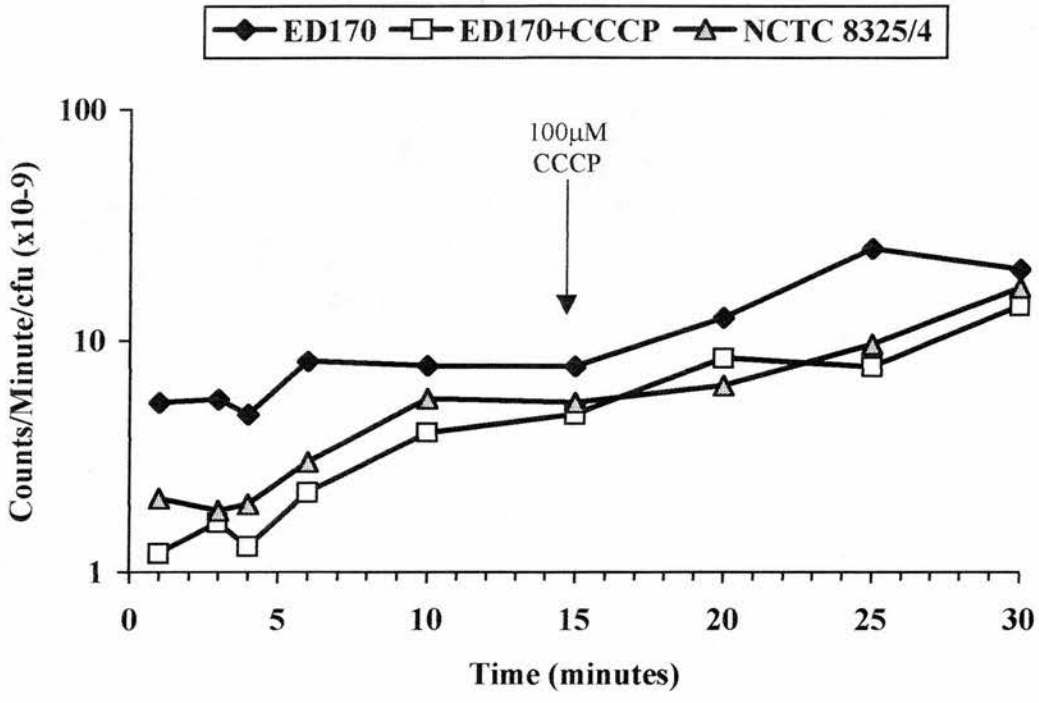


Figure 5.4: Accumulation of radio-labelled moxifloxacin in strain ED209

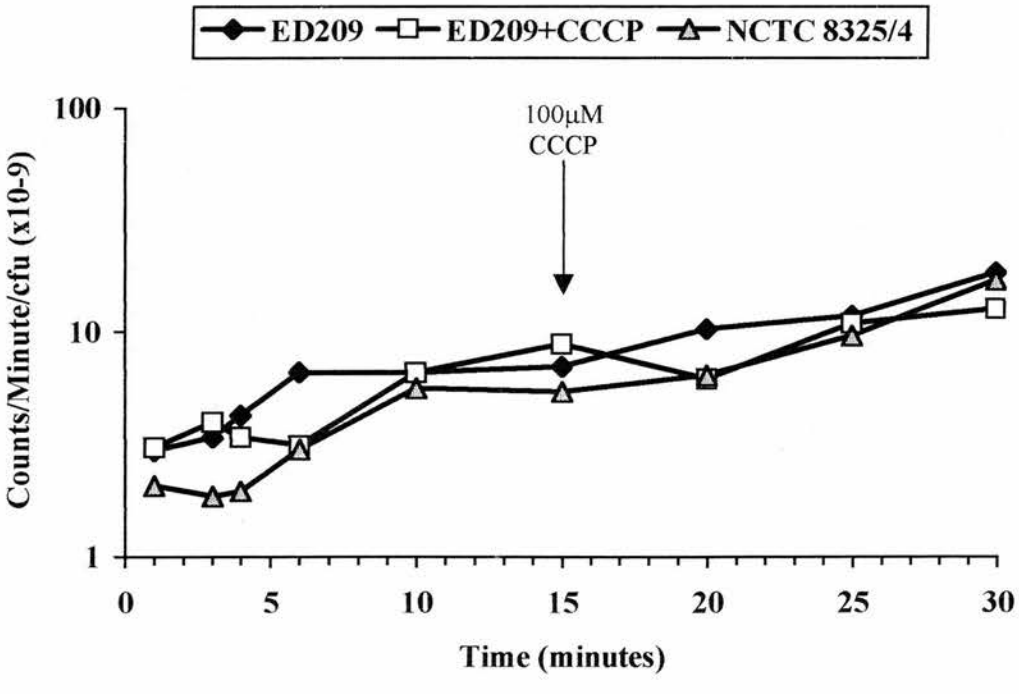


Figure 5.5: Accumulation of radio-labelled moxifloxacin in strain ED216

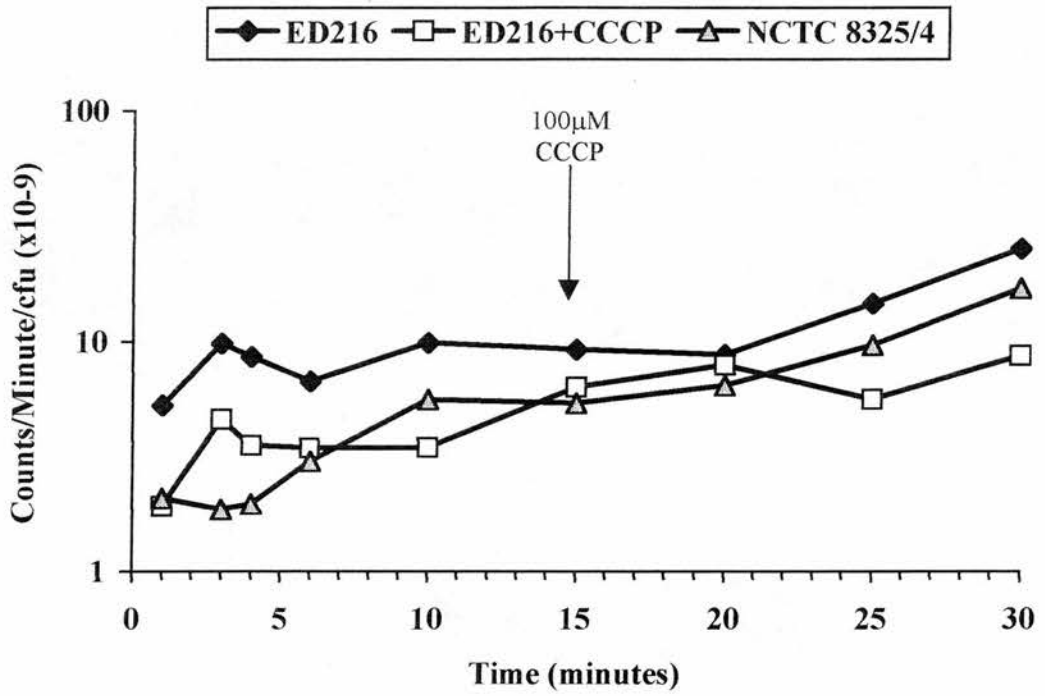


Figure 5.6: Accumulation of radio-labelled moxifloxacin in strain ED223

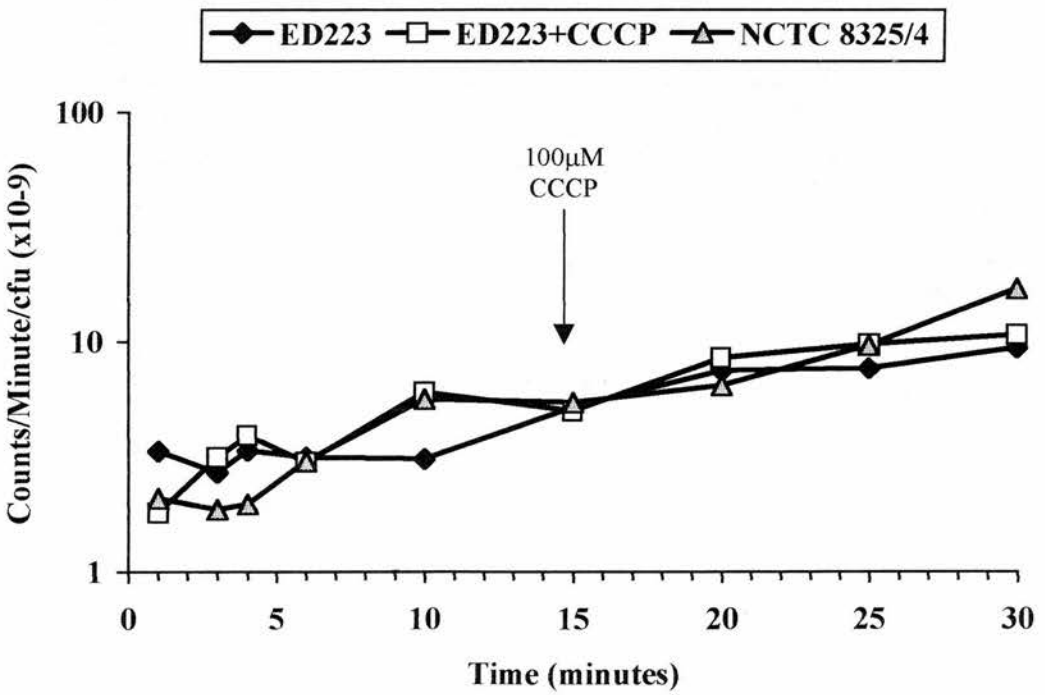
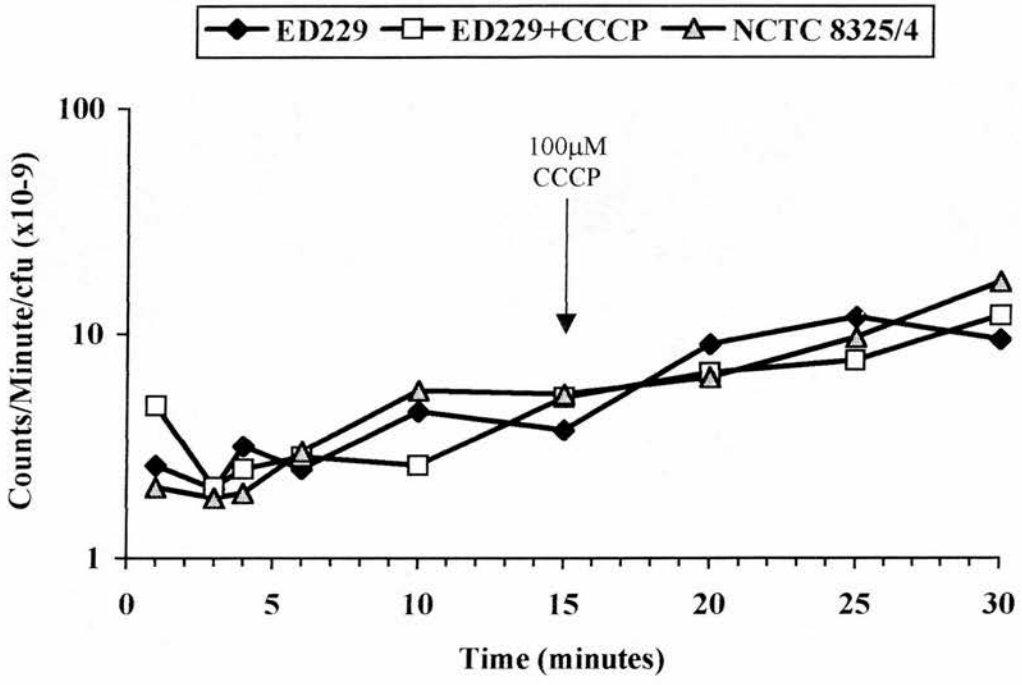


Figure 5.7: Accumulation of radio-labelled moxifloxacin in strain ED229



5.2.3 Washing assay

Since no change in efflux was established using the accumulation assay described in the previous section, a washing assay was employed as an alternative method. The intracellular concentration of radio-labelled moxifloxacin in each strain was measured after multiple washing steps. In strains that over-express an efflux pump such as NorA, export of moxifloxacin from the cells would be expected to be faster than in strains with wild-type expression of NorA.

In Figures 5.8 to 5.12 the first-step mutant strains are compared to the wild-type parent strain NCTC 8325/4.

Figure 5.8: Efflux of radio-labelled moxifloxacin from strain ED170 compared to strain NCTC 8325/4

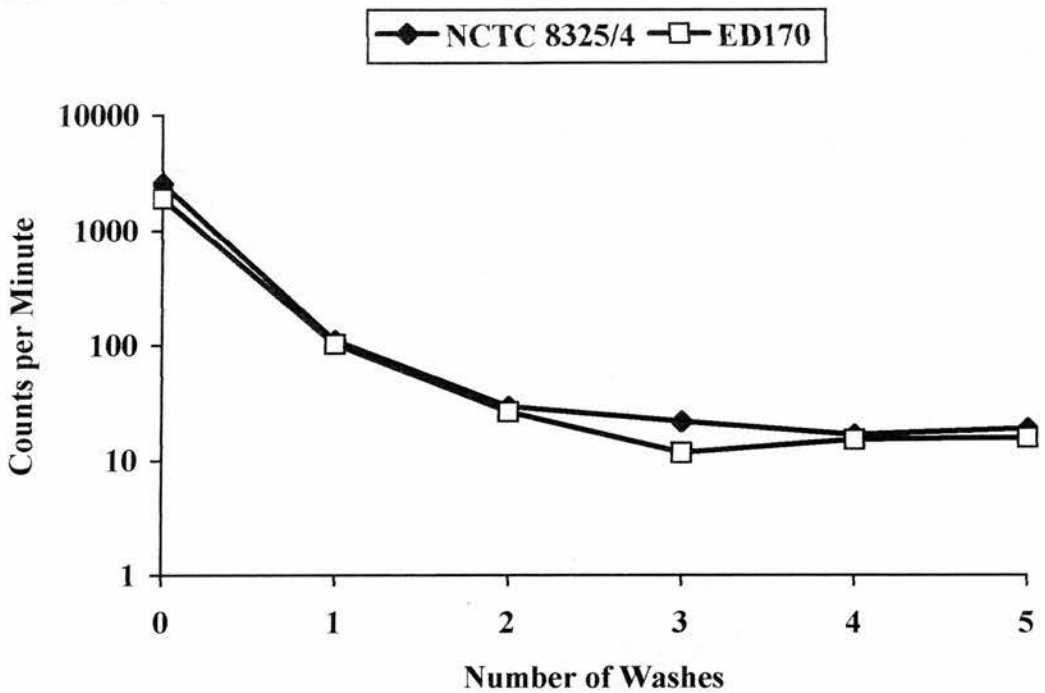


Figure 5.9: Efflux of radio-labelled moxifloxacin from strain ED209 compared to strain NCTC 8325/4

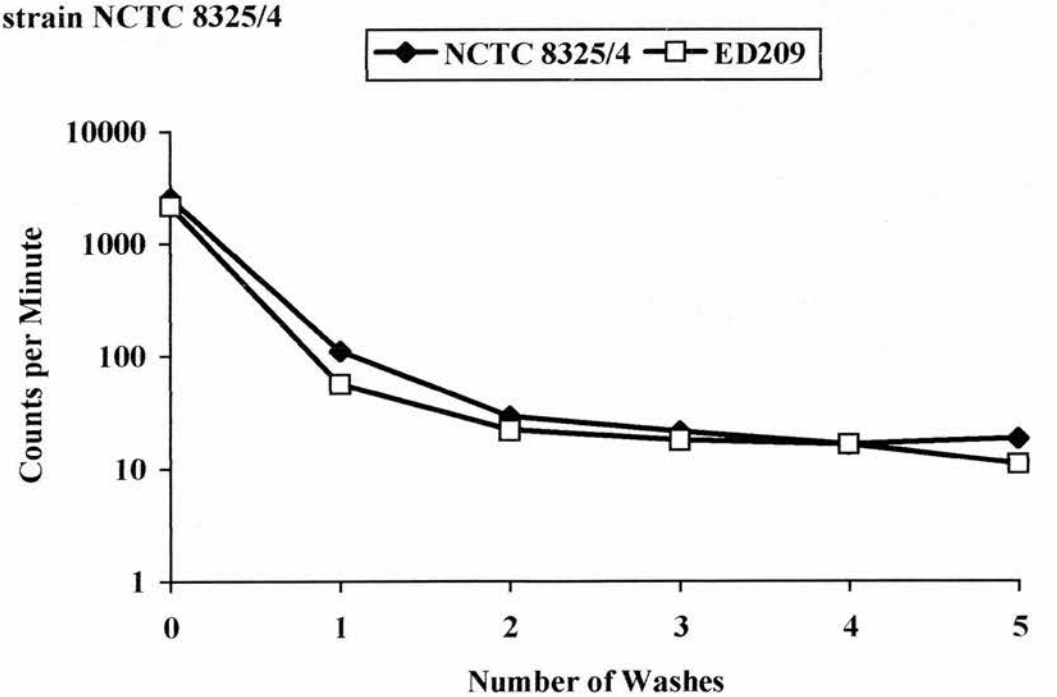


Figure 5.10: Efflux of radio-labelled moxifloxacin from strain ED216 compared to strain NCTC 8325/4

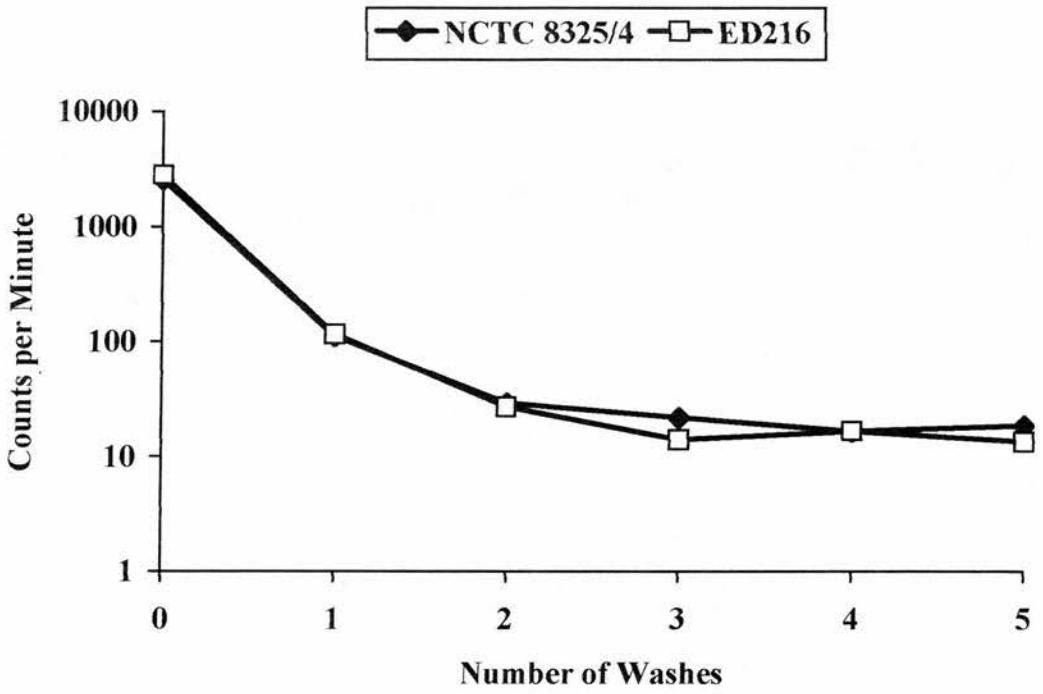


Figure 5.11: Efflux of radio-labelled moxifloxacin from strain ED223 compared to strain NCTC 8325/4

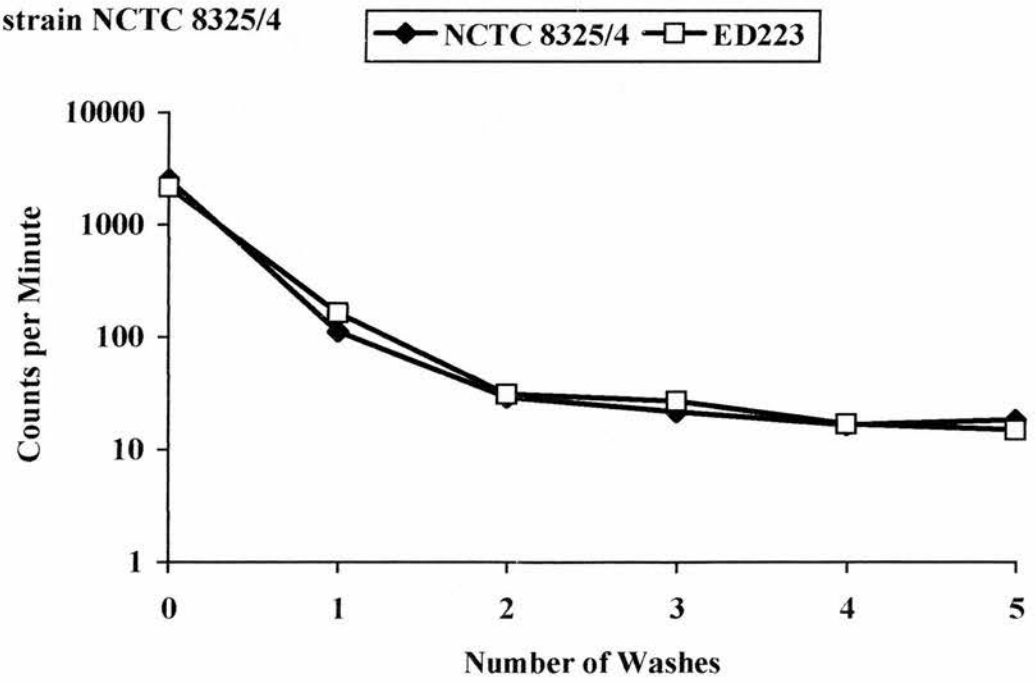
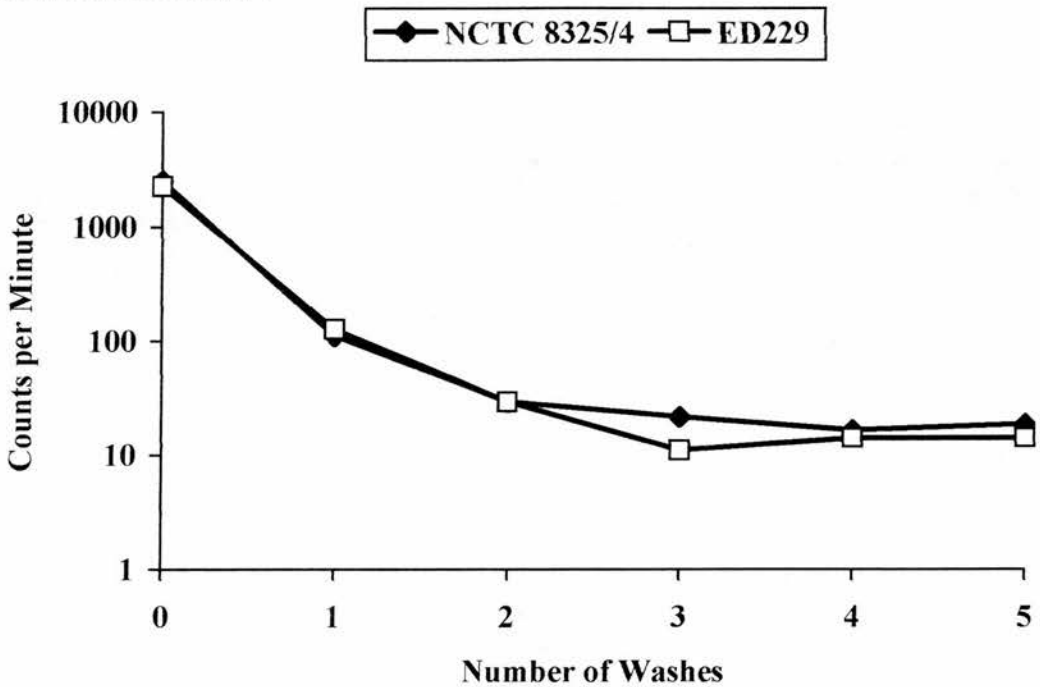


Figure 5.12: Efflux of radio-labelled moxifloxacin from strain ED229 compared to strain NCTC 8325/4



For all strains, the intracellular concentration of moxifloxacin decreases with each subsequent washing step, and eventually levels out to a minimum. The rate of quinolone efflux in all five mutant strains is almost identical to the parent strain NCTC 8325/4. None of the five strains shows significant variation.

Chapter 6: Results – Murine Subcutaneous Abscess Model.

One problem with studying the *in vitro* activity of antimicrobials is relating the resulting data to the situation *in vivo*. The artificial conditions used in the laboratory invariably supply the bacteria with optimal growth conditions in terms of temperature, nutrients and oxygen. However, these conditions may be restricted *in vivo*, resulting in altered growth rate and expression of virulence factors. This, in turn, will have an impact on the spread and severity of disease. The activity of the antimicrobial *in vivo* may be quite different to the activity *in vitro*, due to interactions between biological factors (for example immune system components) with both the infecting bacteria and the antibiotic. Clearly it is therefore important to assess the *in vivo* activity as well as the *in vitro* in the development of any antibiotic, and this may be achieved through the study of animal models of infection and clinical trials.

6.1 Activity in Murine Subcutaneous Staphylococcal Abscess Model

In this study the activity of moxifloxacin and the development of quinolone resistance *in vivo* were investigated in a murine subcutaneous abscess model (Bunce *et al.* 1992).

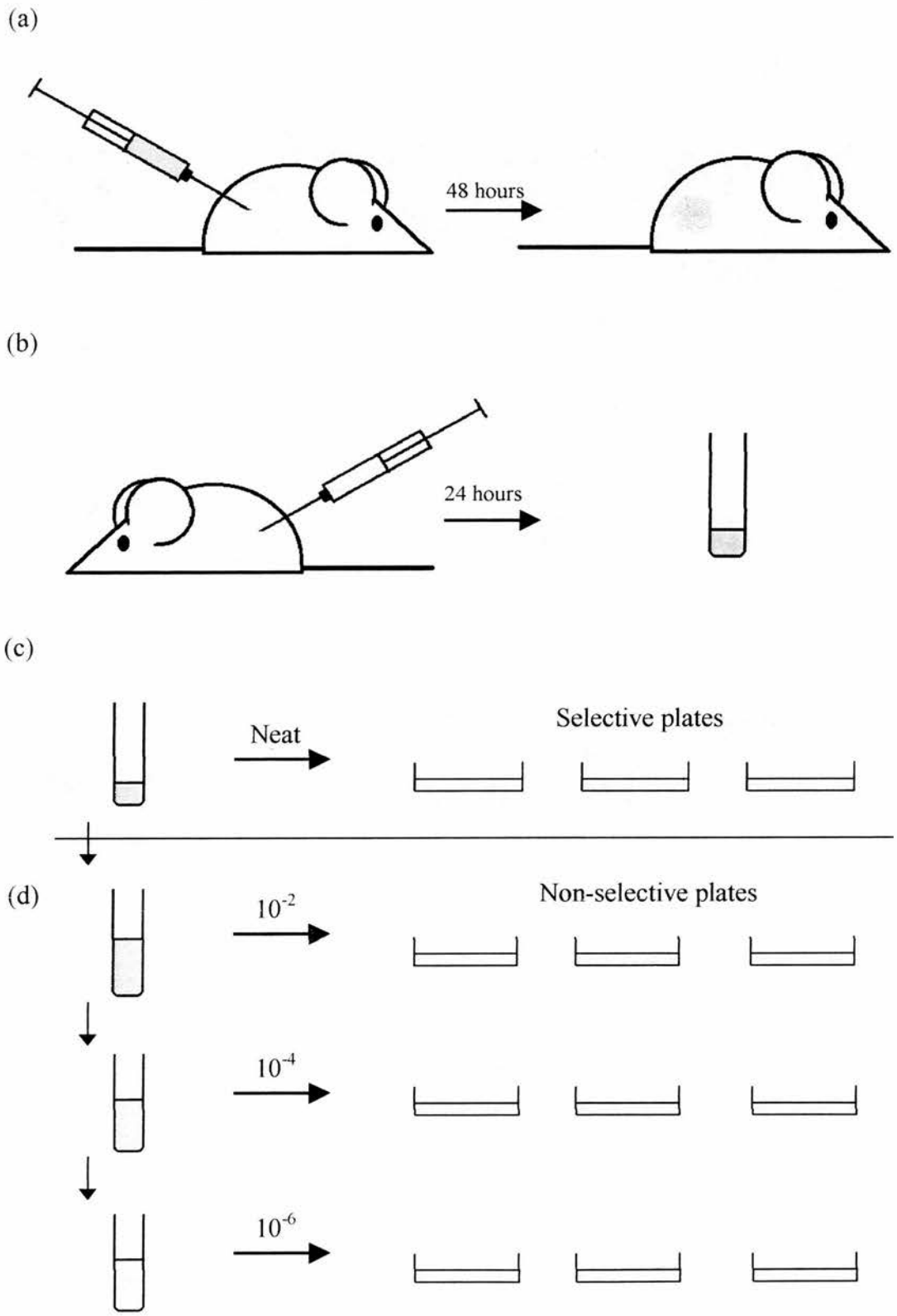
6.1.1 *In vivo* Concentration of Moxifloxacin in Serum and Abscesses

Before the experiment could proceed it was necessary to establish the correct dose of moxifloxacin to give each mouse in order to achieve specific concentrations within the abscesses. Mice were infected on the right flank with *S. aureus* by subcutaneous injection and left for 48 hours to allow abscesses to develop (Figure 6.1a). The mice were then injected with varying concentrations of moxifloxacin (Figure 6.1b). Ocular blood samples were taken after 30 minutes and after three hours the animals were sacrificed and the abscesses excised. The average moxifloxacin concentration within serum samples and abscesses was established by HPLC analysis (Table 6.1).

Table 6.1: Concentration of Moxifloxacin Achieved in Serum and Abscesses

Dose Given (mg/kg body weight)	Serum Concentration (mg/L)	Abscess Concentration (mg/L)
0.00	<0.025	<0.025
0.06	<0.025	0
0.12	0.053	0
0.25	0.063	0.3
0.50	0.135	1.1
1.00	0.300	0.8
2.00	0.550	0.4
4.00	0.700	0.6
8.00	1.950	1.1
16.00	3.550	4.4

Figure 6.1: Summary of the subcutaneous abscess model

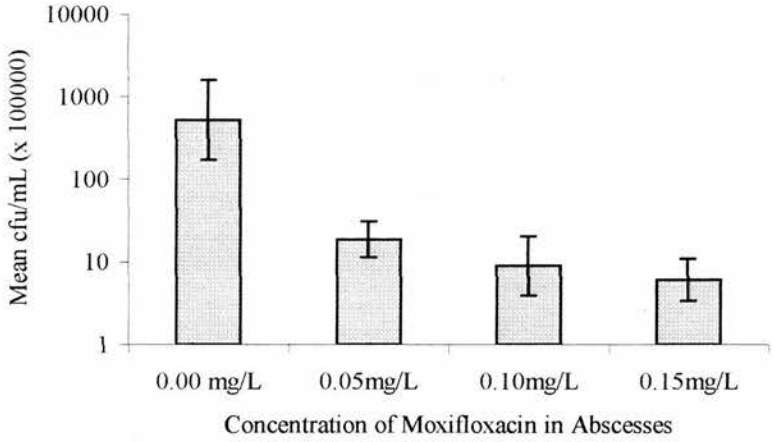


The levels of moxifloxacin in serum and abscesses could be related back to the concentration given in the initial injections. With reference to this data, a suitable dose could be given to achieve the desired multiple of the MIC within abscesses.

6.1.2 *In vivo* Dose Response Curve

The effect of different doses of moxifloxacin on *S. aureus* NCTC 8325/4 in subcutaneous abscesses was investigated. Cohorts of ten mice, infected with wild type strain *S. aureus* NCTC 8325/4 (Figure 6.1a), were dosed with sufficient moxifloxacin to maintain concentrations equivalent to 0, 1, 2 or 4 times the MIC within the abscesses for 24 hours (Figure 6.1b). Thereafter, the number of viable cfus could be determined by plating diluted abscess material onto non-selective MHA plates (Figure 6.1d). Statistical analyses of the data generated are given in Appendix IV.

Figure 6.2: Average viable counts from dosing cohorts given 0, 1, 2 or 4 x MIC doses of moxifloxacin



The average numbers of surviving cfus per dosing group and 95% confidence limits are shown in Figure 6.2. There is a clear dose-response effect with the average number of recoverable cfus decreasing as the given dose of moxifloxacin increases.

6.1.3 *In vivo* Comparison of Moxifloxacin With Other Quinolones

In order to compare the *in vivo* effect of moxifloxacin with other quinolones mice were first infected with *S. aureus* NCTC 8325/4. Every animal in each cohort of ten was given 0.12mg/L (2 x MIC) of moxifloxacin, trovafloxacin or grepafloxacin. The control animals were given phosphate buffered saline only. Statistical analyses of the number of viable cfus recovered are given in Appendix V.

Figure 6.3: Comparison of three quinolones at 0.12mg/L

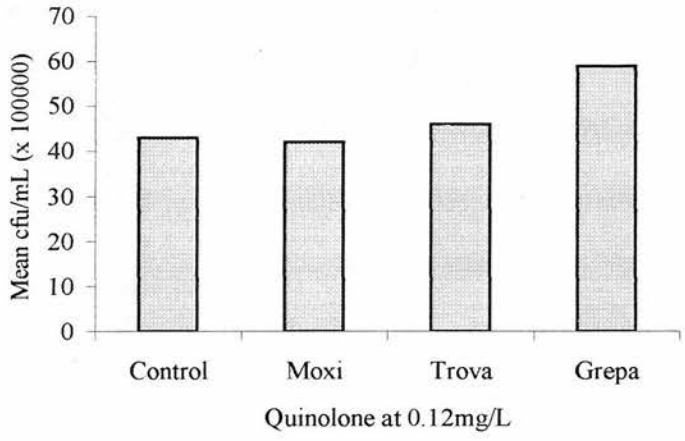
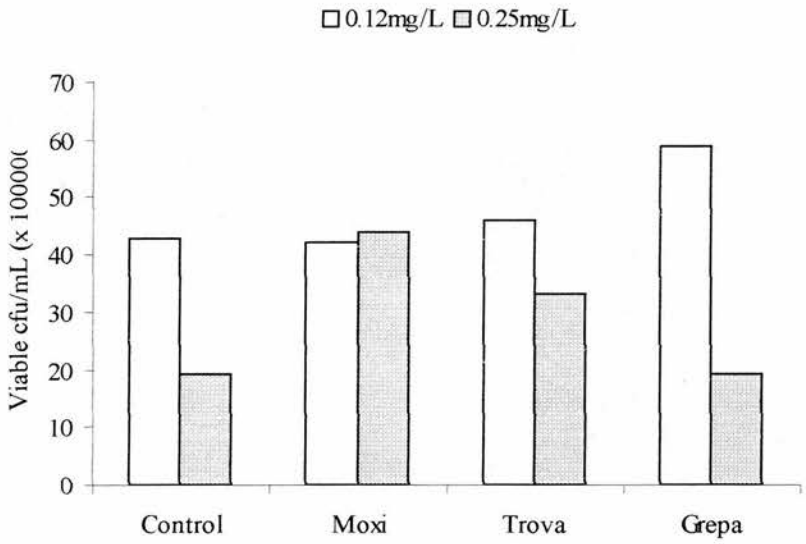


Figure 6.3 shows that the viable count of cfus. Statistical analysis reveals that the viable counts recovered from cohorts given quinolone were not statistically lower

than the control group, although in the previous experiment moxifloxacin at this concentration (0.12mg/L) reduced the survival almost 100-fold (Figure 6.2). Although from the figure it appears that more cfus were recovered from the group treated with grepafloxacin compared to those given moxifloxacin or trovafloxacin, there was no statistically significant difference between the four groups. In order to get a better distinction between the different quinolones for further comparison this experiment was repeated with a starting dose at 0.25 mg/L. Statistical analyses of the number of cfus recovered are given in Appendix VI. Figure 6.4 shows the mean survival of cfus as shaded bars.

Figure 6.4: Comparison of three quinolones at 0.25 mg/L



Although from Figure 6.4 it appears that the numbers of surviving cfus varies between different dosing cohorts, analysis indicates that there are no statistically significant differences between these dosing groups.

6.2 Development of In Vivo Resistance in the Mouse Abscess Model

It is difficult to predict how a new antibiotic is likely to select antibiotic resistance *in vivo* by merely looking at *in vitro* data. However, it is important to know whether a new agent is likely to facilitate rapid selection or spread of resistant strains before it comes into widespread clinical use. A simple model such as the murine subcutaneous staphylococcal abscess model, provides an opportunity to study the *in vivo* development of resistance in response to antibiotic challenge. The propensity of moxifloxacin to select for or promote the development of quinolone resistance in this model was investigated as follows.

6.2.1 First-step Mutants Selected *In vivo*

Mice were infected with *S. aureus* NCTC 8325/4 by subcutaneous injection and treated with moxifloxacin as described previously (section 6.1.2). Mutant selection from abscess material was performed on selective plates containing moxifloxacin at 1, 2 or 4 times MIC in parallel to determining viable counts of surviving cfus (Figure 6.1c). Mutants with MICs of moxifloxacin of between 0.1 and 0.14 mg/L were selected. Table 6.2 details the number of mutants selected and the mutation frequencies from mice given doses equivalent to 0, 1, 2 or 4 times MIC of strain NCTC 8325/4.

From the table it can be seen that fewer moxifloxacin-resistant mutants could be selected from animals treated with moxifloxacin than from the control group, and

that the mutation frequencies did not significantly change as the dose of moxifloxacin increased.

Table 6.2: Mean counts and mutation frequencies of first-step moxifloxacin-resistant mutants selected *in vivo*

Dose (mg/L)	Mean Count	Frequency of Mutation
Control (0.00)	90.0	1.7×10^{-5}
1 x MIC (0.06)	3.0	1.6×10^{-5}
2 x MIC (0.12)	0.4	4.5×10^{-6}
4 x MIC (0.25)	1.1	1.7×10^{-5}

The QRDRs of *gyrA* and *grlA* of five randomly selected mutants (ED405, ED412, ED427, ED460 and ED439) were characterised by PCR amplification and DNA sequencing. All five strains had MICs of moxifloxacin of 0.13 mg/L, however no mutations were detected within the sequenced regions of *gyrA* and *grlA* for any of these first-step mutants. The mutant selection and sequencing results from these first-step mutants were in agreement with previous observations for *in vitro* mutants (see Chapter 4).

6.2.2 Second-step Mutants Selected *In vivo*

Strain ED427 was selected as the parent strain for a subsequent mutation step to select second-step mutants *in vivo*. Abscesses were induced in mice using strain ED427, and mice were treated with moxifloxacin at concentrations of 0, 2, 4 or 8

times the MIC. Although viable cfus could be recovered from abscesses of both treated and untreated animals, no mutants with elevated MICs could be recovered on selective plates at 2, 4 or 8 times the MIC of ED427.

In order to eliminate the possibility that there was a problem with strain ED427, the second-step mutation experiment was repeated with strain ED170, a well characterised first-step mutant strain previously selected *in vitro*. In addition, a double inoculum was used to increase the number of infecting bacteria and thus compensate for slower growth of the mutant strain and any possible decrease in mutation frequency. Mice were treated with an MIC equivalent dose of moxifloxacin (0.12mg/L). Table 6.3 details the total number of strains recovered on selective plates (at 1, 2, 3 and 4 times the MIC) from each dosing group and the mutation frequencies.

Table 6.3: Number of cfus and mutation frequencies of second-step moxifloxacin-resistant mutants selected *in vivo*

Dose (mg/L)	Total cfu Count	Mutation Frequency
Control (0.00)	45	5×10^{-6}
MIC (0.12)	62	6×10^{-6}

The mutation frequencies are similar to those determined for first generation mutant selection. MICs were determined for all 107 strains recovered on selective plates, but only one strain (ED570) demonstrated an increase in resistance with an elevated

MIC of 1.0mg/L. The QRDRs of *gyrA* and *grlA* in this strain were characterised by PCR and DNA sequencing. No mutation was detected within the GrlA protein of topoisomerase IV, however a serine 84 to leucine (TCA to TTA) mutation was found in *gyrA*.

Chapter 7: Results - Mechanisms of Action Against Characterised Quinolone-Resistant Mutants

In Chapter 3 the *in vitro* efficacy and mechanisms of action of moxifloxacin against standard laboratory strains and clinical isolates of *S. aureus* was investigated by time-kill kinetics. Many quinolones have been evaluated by this method, but few studies have also investigated bactericidal activity against characterised quinolone-resistant mutants. During this project a series of step-wise selected mutant strains with resistance to moxifloxacin and other quinolones were characterised for mutations within the QRDRs of *gyrA* and *grlA*. These strains provided a unique opportunity to investigate the effect of specific mutations and combinations of mutations on the bactericidal activity of moxifloxacin.

7.1 Activity of Moxifloxacin Against Characterised Mutants

The minimum concentration of moxifloxacin with the maximum killing activity was established to be 1.0mg/L against standard laboratory strains of *S. aureus* (see section 3.2). This concentration was used to challenge cultures of quinolone-resistant *S. aureus* strains as described previously (section 2.4.5). The number of surviving cfus at 30 minute intervals were determined by viable count.

7.1.1 Activity at 1.0mg/L Against First-step Mutants

Two first-step mutants with MICs of moxifloxacin of 0.12mg/L were challenged with moxifloxacin at 1.0mg/L under three different growth conditions. Strain ED223 had a serine 80 to phenylalanine mutation in the QRDR of *grlA*, but strain ED229 had no mutations in the QRDRs of *gyrA* or *grlA*. Figures 7.1 and 7.2 show the time kill curves determined for strains ED223 and ED229.

Moxifloxacin showed good bactericidal activity against strain ED223 at 1.0mg/L in broth, with less than 1% survival after 120 minutes. At the end of the experiment (210 minutes) only 15% of cfus survived when chloramphenicol was present. Thus the addition of a bacteriostatic concentration of chloramphenicol did not completely remove the killing activity of moxifloxacin. Similarly, challenging the strain in PBS rather than nutrient broth to inhibit cell division did not completely remove killing activity, as only 40% survival was observed after 210 minutes.

A similar pattern of killing was observed for strain ED229 when challenged with 1.0mg/L of moxifloxacin (Figure 7.2). Less than 1% survival was observed in broth after 90 minutes. Some killing activity was retained in the presence of a bacteriostatic concentration of chloramphenicol, with only 32% survival of cfus after 210 minutes. Less than 10% survival was observed when strain ED229 was challenged with moxifloxacin in PBS rather than broth. Figures 7.1 and 7.2 are almost identical to those observed for the wild-type parent strain NCTC 8325/4 (Figure 3.3).

Figure 7.1: Activity of moxifloxacin at 1.0mg/L against strain ED223 in NB, PBS or NB with 20mg/L chloramphenicol

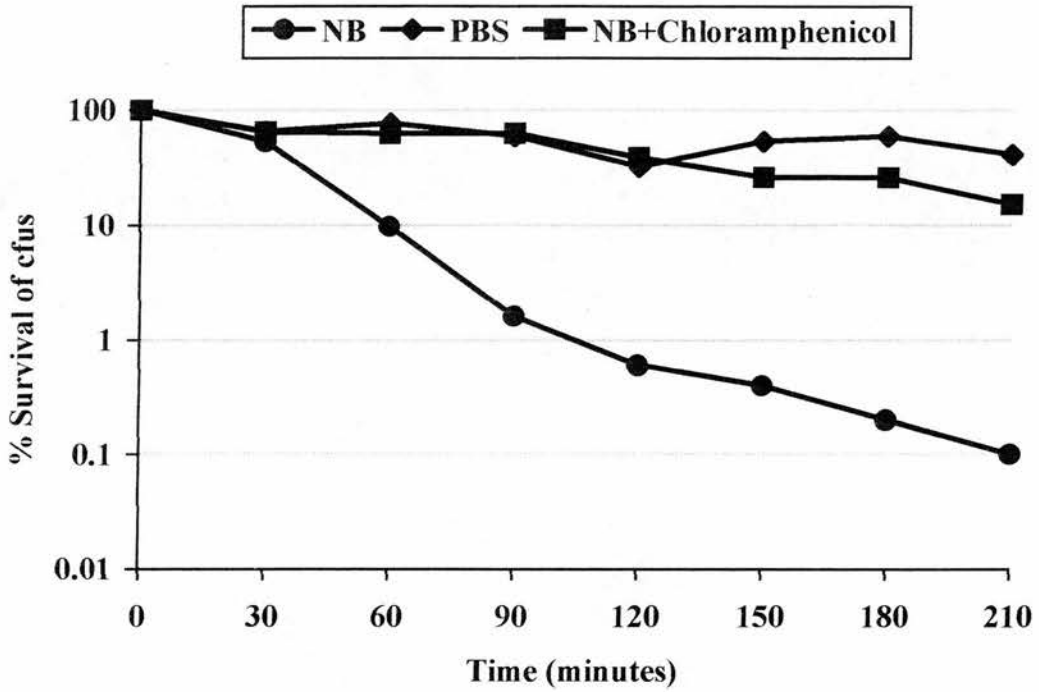
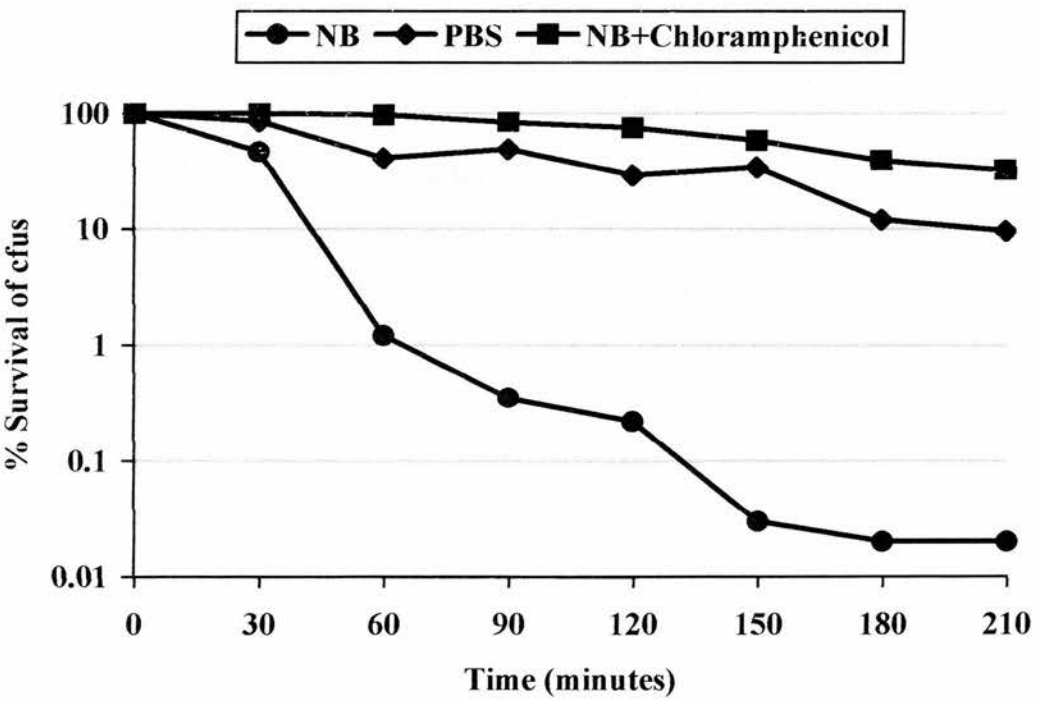


Figure 7.2: Activity of moxifloxacin at 1.0mg/L against strain ED229 in NB, PBS or NB with 20mg/L chloramphenicol



7.1.2 Activity at 1.0mg/L Against Second-step Mutants

The second-step mutants ED258 and ED268 were investigated. The MIC of moxifloxacin for both strains was 0.8mg/L, which was close to the concentration used to challenge them (1.0mg/L). Strain ED258 had a double mutation, with a change at serine 84 to alanine in *gyrA* in addition to the serine 80 to phenylalanine mutation in *grlA* inherited from the parent strain ED223. Strain ED268 had a single mutation at position 84 in *gyrA* with an amino acid change from serine to leucine. Figures 7.3 and 7.4 show the viable counts of surviving cfus for these strains.

Strain ED258 was not killed rapidly, however some killing activity was observed and after 210 minutes only approximately 32% of cfus were recovered (Figure 7.3). The number of surviving cfus remained static in PBS, with counts overall constant at approximately 100%. The presence of chloramphenicol to inhibit protein synthesis had more effect on the kill rate, with the number of surviving cfus decreasing to between 40 and 75%. At 210 minutes there was only 30% survival in the presence of chloramphenicol.

Moxifloxacin was not bactericidal at 1.0mg/L against strain ED268 in broth and more than 180% survival was recorded at 210 minutes (Figure 7.4). However, in PBS some killing was observed and survival decreased to 67% over 210 minutes. Moxifloxacin had a greater killing effect in the presence of chloramphenicol with only 3% survival recorded at 210 minutes.

Figure 7.3: Activity of moxifloxacin at 1.0mg/L against strain ED258 in NB, PBS or NB with 20mg/L chloramphenicol

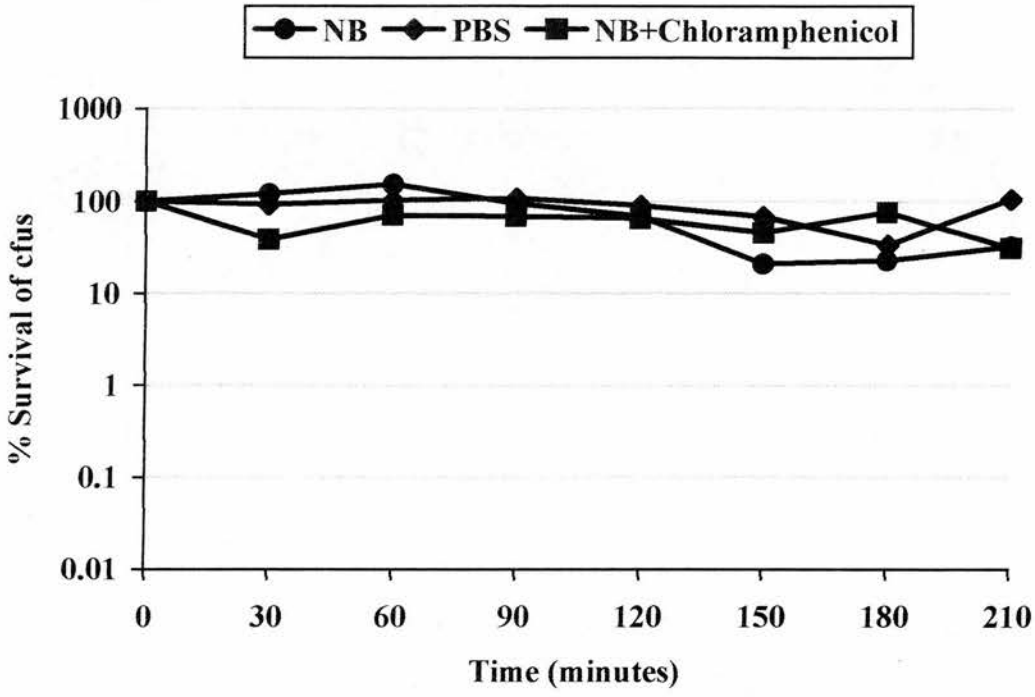
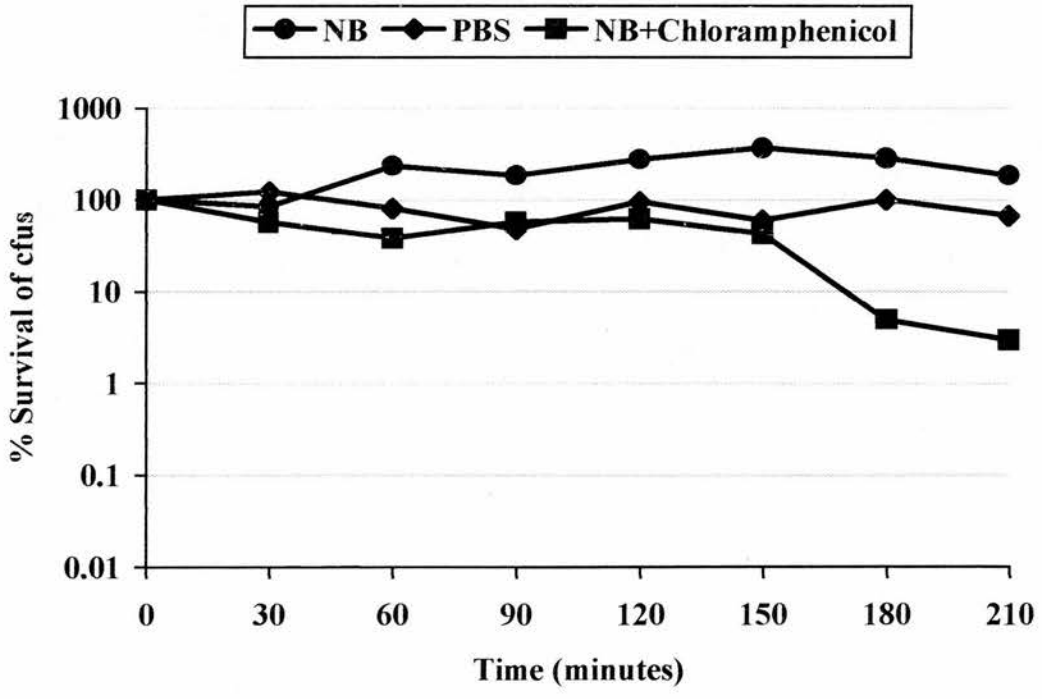


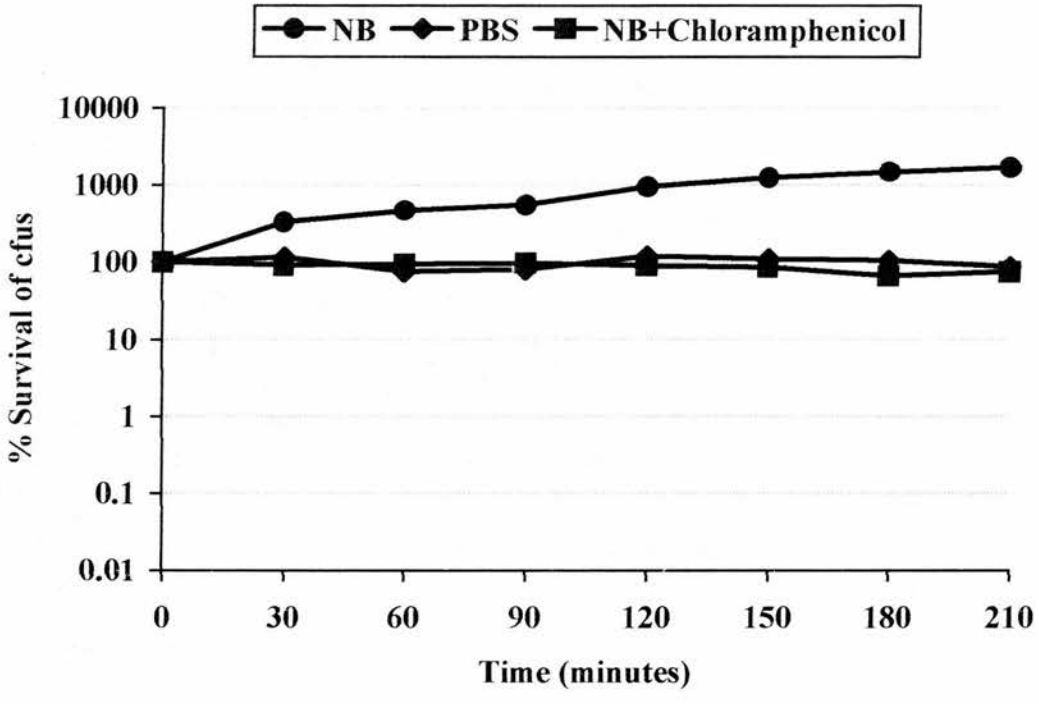
Figure 7.4: Activity of moxifloxacin at 1.0mg/L against strain ED268 in NB, PBS or NB with 20mg/L chloramphenicol



7.1.3 Activity at 1.0mg/L Against a Third-step Mutant

Third-step mutant strain ED360 had a mutational change from glutamic acid to lysine at position 84 in *grlA*, and an additional change of serine 84 to leucine in *gyrA*. The MIC of this strain (4.5mg/L) was higher than the OBC with which it was challenged.

Figure 7.5: Activity of moxifloxacin at 1.0mg/L against strain ED360 in NB, PBS or NB with 20mg/L chloramphenicol



From Figure 7.5 it is clear that the OBC has no killing effect on this strain, and after 210 minutes survival was greater than 1600%. Limiting active cell division by incubation in PBS rather than broth prevented cell multiplication – viable counts remained at approximately 100% throughout the experiment – but no bactericidal

activity of moxifloxacin was observed. In the presence of chloramphenicol minimal killing was observed after 150 minutes, with 75% survival at 210 minutes.

7.1.4 Activity at 10mg/L of Moxifloxacin Against Selected Strains

The OBC of moxifloxacin, as established against sensitive standard strains is 1.0mg/L. This concentration is considerably higher than the corresponding MICs for these strains. However, although this concentration is greater than the MIC of the first-step mutant strains, it is close to the MIC of the second-step mutants and lower than the MIC of the highly resistant third-step strains. Since a challenge with 1.0mg/L of moxifloxacin would not be bactericidal against all of these mutant strains, one strain from each mutation step was subsequently challenged with 10mg/L in broth, PBS or broth including chloramphenicol.

Figure 7.6 shows the time-kill curves for first-step mutant strain ED229 after challenge with 10mg/L of moxifloxacin. As expected, the killing activity of 10mg/L moxifloxacin in broth was rapid with less than 0.01% survival after 210 minutes. Moxifloxacin had a greater killing effect against bacteria in PBS and in broth containing chloramphenicol than at 1.0mg/L (Figure 7.2), with less than 1% survival of cfus after 180 and 120 minutes respectively.

Figure 7.6: Activity of moxifloxacin at 10mg/L against strain ED229 in NB, PBS or NB with 20mg/L chloramphenicol

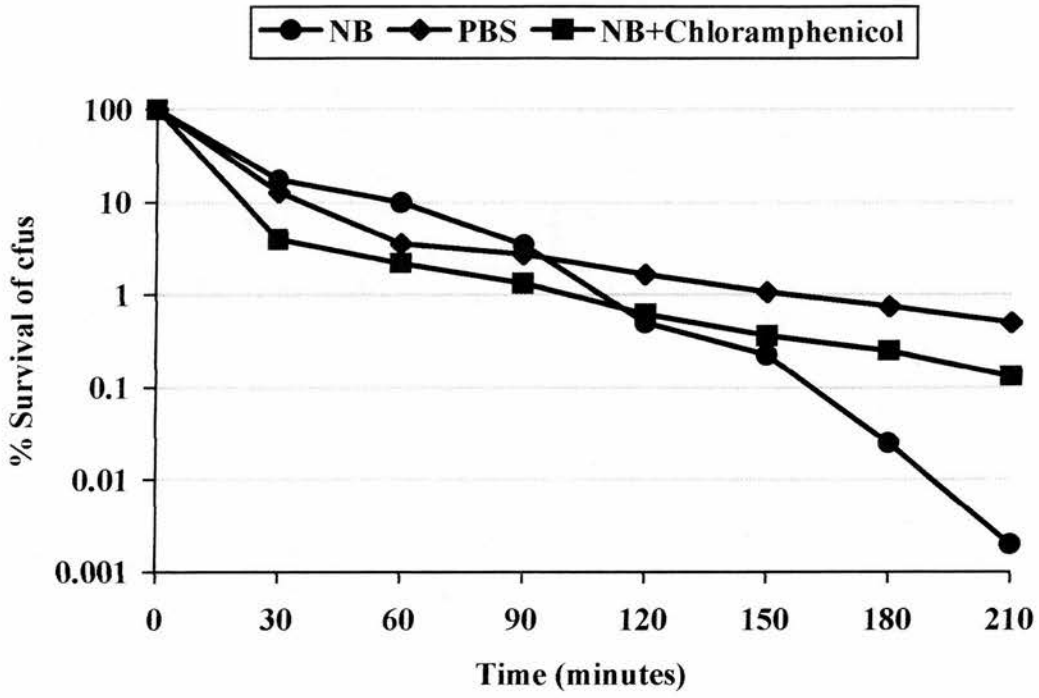
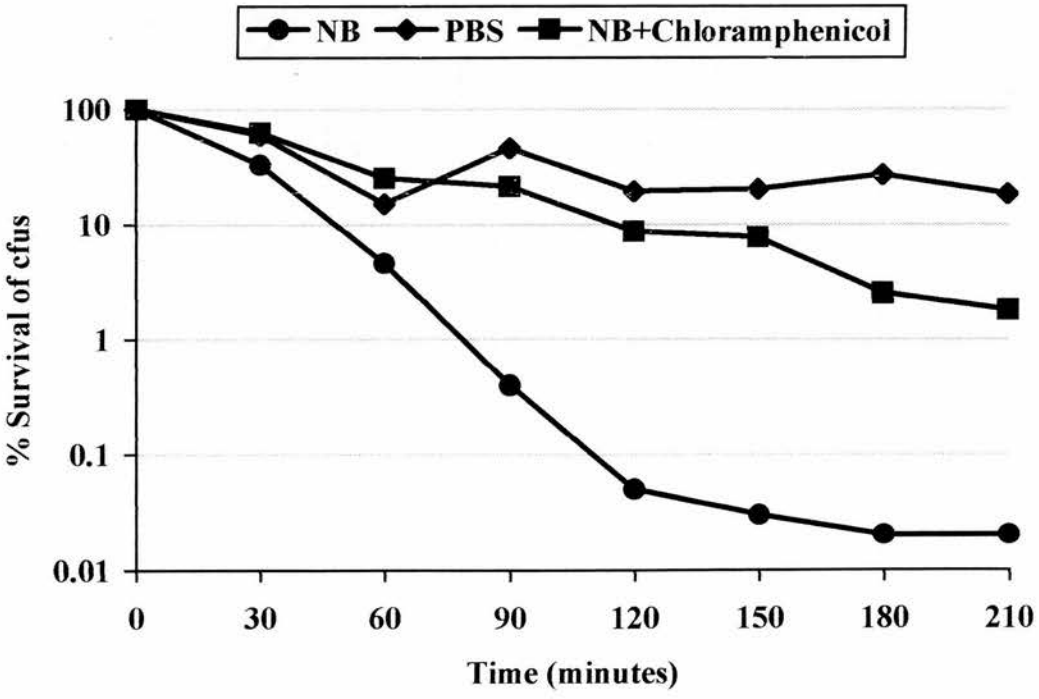


Figure 7.7: Activity of moxifloxacin at 10mg/L against strain ED268 in NB, PBS or NB with 20mg/L chloramphenicol



Moxifloxacin was not bactericidal against strain ED268 at a concentration of 1mg/L (Figure 7.4), however the time-kill curve in Figure 7.7 shows that at 10mg/L in broth there was rapid bactericidal activity. Bacterial survival after 210 minutes in the presence of chloramphenicol was similar to that determined at 1.0mg/L (Figure 7.4). However, survival in PBS at 10mg/L was reduced with approximately 18% survival after 210 minutes compared to 67% when challenged with 1mg/L.

Figure 7.8: Activity of moxifloxacin at 10mg/L against strain ED360 in NB, PBS or NB with 20mg/L chloramphenicol

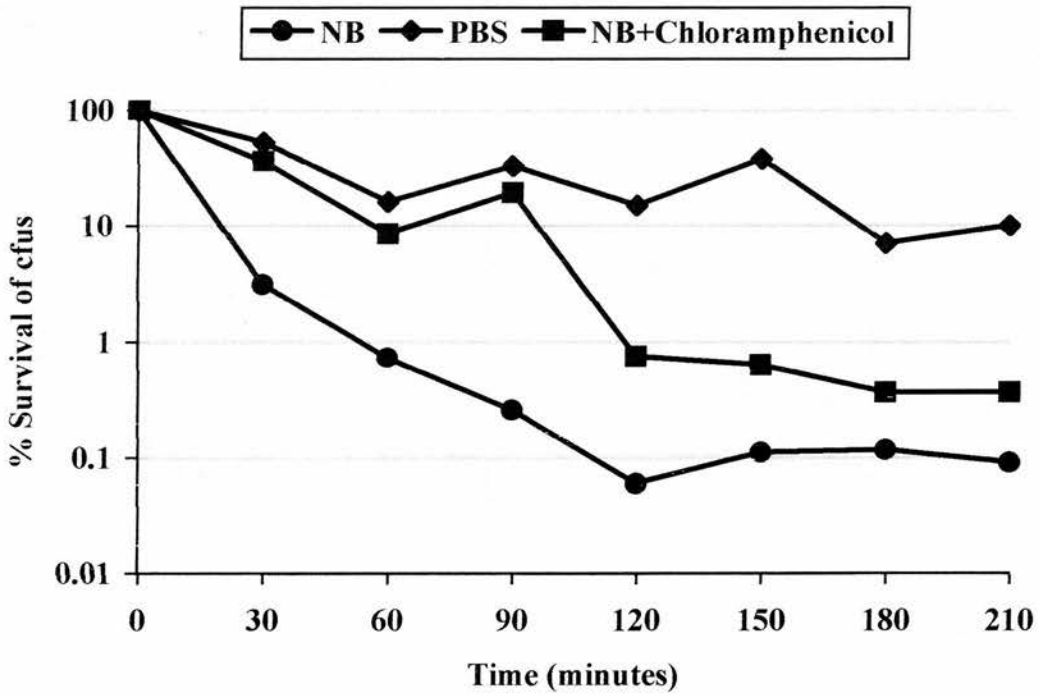


Figure 7.8 shows the survival of the third-step mutant strain ED360 after challenge with 10mg/L of moxifloxacin. Increasing the concentration of moxifloxacin from 1mg/L to 10mg/L restored bactericidal activity against this strain, with less than 0.1% survival after 210 minutes in broth. Bactericidal activity was also restored

against bacteria in PBS or in broth containing chloramphenicol, with 10% and less than 1% survival of cfus under these conditions by 210 minutes respectively.

Chapter 8: Discussion

As the incidence of infections due to multi-drug-resistant pathogens has continued to increase, the need for new antibiotics with which to treat such infections has become more urgent. During the 1990s, several new generation quinolones with a broader spectrum of activity encompassing Gram-positive as well as Gram-negative organisms have been developed. Unfortunately, some of these agents have already been withdrawn, or restricted in their clinical use, due to excessive toxic side effects (Nightingale, 1999). Moxifloxacin is a new 8-methoxyquinolone with good *in vitro* efficacy against both Gram-positive and Gram-negative organisms (Alcala *et al.* 1999; Balfour and Wiseman, 1999; Al-Nawas and Shah, 1998; Klugman and Capper, 1997; Aldridge and Ashcraft, 1996; Dalhoff *et al.* 1996; Kitzis *et al.* 1996; Wise *et al.* 1996), and clinical trials indicate that adverse effects are infrequent (von Keutz and Schlüter, 1999; Man *et al.* 1999; Sullivan *et al.* 1999; Siefert *et al.* 1999a; Stass *et al.* 1998; Kubitza *et al.* 1996).

Prior to the development of modern molecular biology techniques, the activity of quinolones was often evaluated through study of time-kill kinetics and mechanisms of action (Smith, 1984). In the first instance a dose response curve may be constructed by challenging bacteria in liquid culture with increasing concentrations of quinolone. Traditionally, the dose response curve of killing by quinolones is easily recognised by its characteristic biphasic shape. The survival of bacteria

decreases with increasing concentration of quinolone to a minimum point, the OBC. Survival subsequently improves with further increases in quinolone concentration and this has been suggested to be caused by quinolone inhibition of protein synthesis required for the killing action (Smith, 1984). The results presented in this thesis show that moxifloxacin did not demonstrate the classic dose response curve observed for older quinolones in that survival decreased to a minimum point but did not subsequently increase again. This minimum point was approximately 1.0mg/L. Pirapatrungsuriya (1998) observed a similar non-biphasic pattern of killing when challenging *S. aureus* with ciprofloxacin. Although no multiplication was seen at concentrations greater than 1.0mg/L, moxifloxacin was clearly bacteriostatic rather than bactericidal as some cfus were recoverable. It is possible that bacteria that survived at high concentrations may have developed a mutation, such as enhanced efflux, conferring sufficient resistance to allow survival. However, since efflux mutations have only been correlated to low levels of quinolone resistance (Kaatz and Seo, 1995; Kaatz *et al.* 1993) it seems unlikely that this mechanism would account for survival against high moxifloxacin concentrations. It is also possible that the duration of this experiment was insufficient to allow an increase in the bacterial population, and that given longer incubation times the number of surviving cells could eventually begin to increase as resistant strains multiply. Quinolones are known to inhibit cell division and septation, causing cell filamentation. This process is associated with disruption in topoisomerase IV function although the underlying mechanisms have not been fully determined (Maxwell and Critchlow, 1998). The bacteria surviving after exposure to high quinolone concentrations may therefore

have been strains that survived by becoming filamentous, enabling cell survival but not multiplication. New quinolones such as moxifloxacin may have different molecular targets compared to older agents, and this may account for variation in the classic biphasic response curve. Interestingly, moxifloxacin does exhibit a classic biphasic dose response against the Gram-negative bacterium *Acinetobacter baumannii* (Higgins *et al.* 1999).

Survival of quinolone sensitive strains (both laboratory standards and clinical strains) was reduced to less than 1% by 210 minutes when challenged with 1.0mg/L. Although resistant strains ED3 and ED5 survived after challenge with 1.0mg/L, less than 1% survived after challenge with 4.0mg/L, a concentration equivalent to two times the MIC for these strains. Pharmacokinetic data for moxifloxacin indicate that serum concentrations of 4.73mg/L are achievable *in vivo*, so treatment of infection with resistant strains such as these should not present a therapeutic problem (Stass *et al.* 1998).

At least four bactericidal mechanisms, termed A, B, B′ and C, have been described for the quinolones (Smith and Zeiler, 1998). However, not all mechanisms are exhibited by all quinolones, and some quinolones demonstrate different killing mechanisms against different organisms (Lewin and Smith, 1988). Quinolone killing by mechanism A, found for all quinolones, requires active protein synthesis and cell division and is observed when challenging a strain in broth. Although the molecular basis of mechanism A has not been fully elucidated, it has been suggested that it is

related to the blocking of replication by the formation of cleavable complexes between a quinolone and DNA gyrase on DNA strands (Chen *et al.* 1996). Moxifloxacin was able to kill by mechanism A in all cases. The growth of strains ED3 and ED5 was not inhibited by subinhibitory concentrations of moxifloxacin (1.0mg/L), but increasing the moxifloxacin concentration restored bactericidal activity. It is clear that moxifloxacin is still able to kill these strains by mechanism A albeit at higher concentrations.

Mechanisms B, B′ and C, do not require both protein synthesis and cell division and can be observed when either or both are inhibited. The data in this thesis show that killing by moxifloxacin is observed irrespective of the presence of protein synthesis or cell division against strains NCTC 6571, NCTC 8325/4 and E3T. These data indicate killing by another mechanism is present in addition to mechanism A. This is confirmed in the case of clinical strains ED7 and ED9 which show similar patterns of killing kinetics. To date mechanisms B′ and C have only been observed in Gram-negative organisms with older agents (Lewin and Amyes, 1990; Lewin *et al.* 1989; Ratcliffe and Smith, 1985). Mechanism B acts independently of RNA or protein synthesis and may be related to dissociation of the DNA gyrase subunits. This mechanism has been described for several quinolones including new agents similar to moxifloxacin such as sparfloxacin (Lewin *et al.* 1992). Mechanism C has been suggested to relate to the trapping of cleavable complexes between a quinolone and topoisomerase IV (Maxwell and Critchlow, 1998). Since moxifloxacin targets topoisomerase IV as well as DNA gyrase in *S. aureus* this mechanism of action may

be present. In this thesis, mechanisms of action in addition to mechanism A were investigated only by the addition of a bacteriostatic concentration of chloramphenicol to the test strain (or incubation in PBS) at the same time that moxifloxacin was added. However, while this shows the presence of an additional mechanism of action, studies with inhibitor combinations instead of a single agent are necessary to conclusively differentiate between mechanisms B, B′ and C. Studies by Pirapatrungsuriya (1998) showed that time-kill studies may not always be an accurate method of identifying the presence of mechanism B, since for *E. coli* the effect of the inhibitors chloramphenicol and rifampicin vary greatly, depending whether they are added before, together with or after the quinolone. Therefore, although moxifloxacin exhibits a second mechanism of action in addition to mechanism A, it is not possible to conclude whether this mechanism is B, B′ or C from the data given in this thesis. Indeed the same is likely to be true for all studies that have reported these mechanisms but have not altered the timings of the protein and RNA synthesis inhibitors.

The activity and mechanisms of quinolone action against characterised quinolone-resistant mutants have rarely been investigated although a significant amount of work has been published about standard sensitive strains. Mutations within DNA gyrase or topoisomerase IV may effect the degree of DNA supercoiling and hence the rates of transcription (Bagel *et al.* 1999). This, in turn, will have an impact on protein expression and hence growth rate or doubling times, which may be detectable by time-kill experiments. A specific mutation that extends the survival of

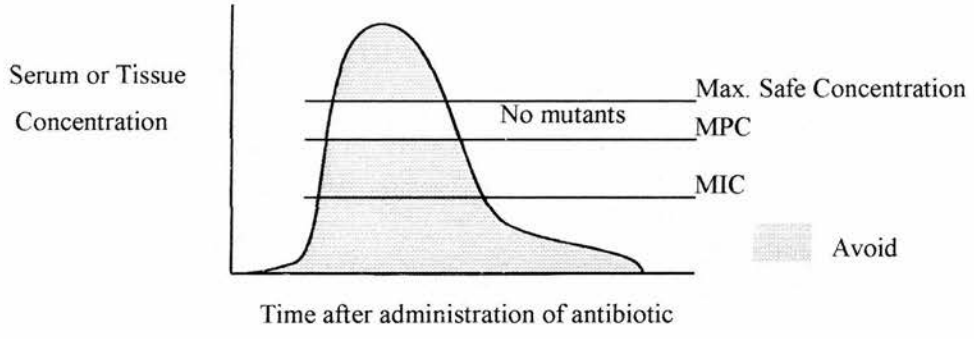
a bacterium longer than other mutations, i.e. long enough for subsequent changes to occur leading to higher levels of resistance, will give a selective advantage. If this occurs *in vivo*, strains with specific mutations will tend to dominate. In Chapter 7 the results of a study to investigate the effect of specific mutations in *gyrA* and *grlA* on the time-kill kinetics of moxifloxacin are given. As expected, moxifloxacin was rapidly bactericidal at 1.0mg/L against first-step mutant strains ED223 and ED229, for which this concentration was much greater than the MIC. However, only the second-step strain ED258 was killed at 1.0mg/L, with strain ED268 surviving and multiplying, and third-step strain ED360 was not killed at 1.0mg/L. It is not clear why strain ED258 (with both a *gyrA* and a *grlA* mutation) should be killed more efficiently than strain ED268 (which had only a *gyrA* mutation). However, it may be that an efflux mutation, which ED268 may have acquired at the primary mutation step, confers an advantage over the *grlA*80 mutation of ED258 by decreasing the intracellular concentration of quinolone. This could reduce the degree of quinolone-induced DNA cleavage and allow the SOS system to repair the damage and prolong survival. Since prolonged survival increases the chance of a new mutational event occurring, which will confer greater quinolone resistance, it would be interesting to isolate and sequence strains from the end point of this experiment to determine whether additional mutations have arisen. When strains ED229, ED268 and ED360 were subsequently challenged with a 10-fold higher concentration of moxifloxacin, killing activity was restored against highly resistant strains with survival of all strains decreasing to less than 0.1% after 210 minutes. In all cases killing was rapid and, given the relatively short time frame of these experiments,

there were no significant differences between the survival times of different strains. Increasing the duration of quinolone challenge would give a better idea of the ability of strains to recover from challenge and the propensity for long term survival, and has been done in some studies (Thomson and Sanders, 1998).

From these results there is no evidence to suggest that the specific mutations in these strains affect the growth rate *in vitro*, or that certain strains will survive longer than others after quinolone challenge. However, it is unclear how these data may relate to survival in the clinical situation, as the effect of these mutations on the *in vivo* pathogenicity of these strains is unknown. One recent study of the activity of a variety of experimental quinolones compared to ciprofloxacin, against sensitive and resistant strains of *S. aureus*, found that those with a C-8-methoxy group were particularly effective against first-step mutants carrying only a *grlA* mutation (Zhao *et al.* 1998). Clearly a quinolone, which is better able to eradicate first-step mutants, could have significant benefits in preventing the development of high-level resistance if used appropriately in the clinical situation. This study also found the presence of a C-8-methoxy group conferred better activity against non-growing cells than that observed for quinolones lacking this group. A reduction in the number of viable static-phase cells also minimises the possibility of a mutation occurring (Zhao *et al.* 1998). In addition to this, Dong *et al* (1999) investigated the relationship between quinolone structure and resistance by assessing the effect of concentration on the recovery of mutants. By applying 10^{10} cells to agar plates containing various concentrations of quinolones, they determined a concentration above the MIC at

which no mutants could be recovered. This concentration was termed the mutant prevention concentration (MPC), and seems to be lowered by the presence of a C-8-methoxy group. The potential advantage of determining the MPC is illustrated in Figure 8.1. In the clinical situation, concentrations represented by the shaded area should be avoided: concentrations below the MIC do not prevent cell growth and promote the selection of mutants; concentrations between the MIC and the MPC also allow growth of resistant mutants; concentrations above the maximum safe concentration are toxic to the patient. Establishing the MPC may be a useful method of determining agents least likely to select resistance (Dong *et al.* 1999).

Figure 8.1: Diagram representing the MPC



Resistance to the quinolones in *S. aureus* is known to develop in a step-wise manner through sequential mutations in the genes encoding DNA gyrase, topoisomerase IV or efflux pumps. Ferrero *et al* (1995; 1994) showed that topoisomerase IV is a quinolone target in *S. aureus* when mutants are selected in a step-wise manner. With ciprofloxacin as the selective agent, *grlA* mutations were selected before *gyrA* mutations, leading to the assumption that topoisomerase IV is the primary target of

quinolones in *S. aureus*. However, ciprofloxacin primarily targets Gram-negative organisms unlike moxifloxacin that also has good anti-Gram-positive activity, therefore the targets of moxifloxacin could be different from ciprofloxacin. In this study the step-wise development of resistance to moxifloxacin was investigated to establish the progression of mutations and the primary molecular target in *S. aureus*. Sensitivity testing confirmed that there was cross-resistance between different quinolones although strains with identical MICs of moxifloxacin did not always have identical MICs for all other quinolones tested. However, for some quinolones the pattern of sensitivity correlated with the presence of mutations in *gyrA* and *grlA*: second-step strains ED258 and ED261 had two mutations (*gyrA* and *grlA*), and both showed higher MICs of ciprofloxacin and trovafloxacin than the other second-step mutants. Mutations in *gyrA* were selected before mutations in *grlA* in the majority of strains. Thus it is valid to suggest from these data that DNA gyrase is the primary target of moxifloxacin, rather than topoisomerase IV, because it is the primary site of resistance mutations.

Ferrero *et al* (1995) proposed topoisomerase IV as the primary target of ciprofloxacin in *S. aureus*, because *grlA* mutations always preceded *gyrA* mutations. However, in this thesis strains selected with ciprofloxacin showed the same pattern of mutation as those selected with moxifloxacin, with mutations found in DNA gyrase and not in topoisomerase IV in the majority of strains. Ferrero *et al* (1995) concluded that mutations in *grlA* must be a prerequisite to mutations in *gyrA*, however the results of this thesis clearly show that this is not necessarily true.

Table 8.1: Summary of published mutation studies in comparison to data described in this thesis

Study	Quinolone/ Organism	Mutation in gyrase or topoisomerase IV?			Suggested Primary Target
		1st Step	2nd Step	3rd Step	
This Thesis	moxifloxacin/*S. aureus*	1 strain *grlA*	2 strains *grlA* + *gyrA*	5 strains *gyrA* +	DNA gyrase
		4 strains other*	3 strains *gyrA* + other*	*grlA* + other*	
	ciprofloxacin/*S. aureus*	1 strain *grlA*	2 strains *gyrA* + other*	-	DNA gyrase
		6 strains other*	3 strains other*	-	
(Ferrero *et al.* 1995)	ciprofloxacin/*S. aureus*	6 strains *grlA*	3 strains *grlA* + efflux	2 strains *grlA* +	topoisomerase IV
			1 strain *grlA* + *gyrA*	*gyrA* + efflux	
(Pan and Fisher, 1997)	sparfloxacin/*S. pneumoniae*	4 strains *gyrA*	4 strains *gyrA* + *parC*		DNA gyrase + topoisomerase IV
(Pan and Fisher, 1998)	clinafloxacin/*S. pneumoniae*	5 strains *gyrA*	5 strains *gyrA* + *parC*	3 strains *gyrA* +	DNA gyrase +
		1 strain *gyrB*		*gyrA* + *parC*	topoisomerase IV
(Kenny *et al.* 1999)	sparfloxacin/*M. hominis*	2 strains *gyrA*	5 strains *gyrA* + *parC*		DNA gyrase
	ofloxacin/*M. hominis*	2 strains *parC*	3 strains *parC* + *gyrA*		topoisomerase IV

* Indicates undetermined mutations that are not within the QRDRs. These may be efflux mutations.

Sulavik and Barg (1998) stated that a *gyrA* mutation without an accompanying *grlA* mutation can have no effect on quinolone susceptibility and reasoned that this was why no such mutations have been identified amongst first-step *in vitro* mutants. Although *gyrA* mutations have not been identified in first-step *in vitro* mutants, it is clear from this thesis that *gyrA* mutations can have a significant impact on strain sensitivity in the absence of *grlA* mutations. From these data, DNA gyrase appears to be the primary target of both moxifloxacin and ciprofloxacin in *S. aureus*. Table 8.1 gives a summary of the results of several key mutation studies. *In vitro* studies of *S. pneumoniae* have indicated that newer quinolones such as sparfloxacin and clinafloxacin have DNA gyrase and topoisomerase IV as dual targets (Varon *et al.* 1999; Pan and Fisher, 1998; Pan and Fisher, 1997). In another recent study the primary quinolone target in *Mycoplasma hominis* was found to vary depending on the selective agent – sparfloxacin selected gyrase mutations but ofloxacin selected topoisomerase IV mutations from the same parent strain (Kenny *et al.* 1999). Evidently these studies and the evidence of this thesis disprove the theory that all quinolones primarily target DNA gyrase in Gram-negative organisms and topoisomerase IV in Gram-positive organisms.

The fundamental question, however, is whether these results generated *in vitro* with laboratory strains accurately reflect the progression of resistance in clinical strains. Clearly it is difficult to assess the step-wise development of high-level resistance *in vivo*, as many clinical strains carry multiple mutations. Clinical studies generally show that the majority of strains with high-level resistance have at least one mutation

in *gyrA* and one in *grlA*, and often multiple mutations in both genes (MunozBellido *et al.* 1999; Schmitz *et al.* 1998; Takahashi *et al.* 1998; Wang *et al.* 1998; Deplano *et al.* 1997). Schmitz *et al* (1998) found one strain out of 116 isolates with a *gyrA* mutation alone, and this reflects the rarity of these mutants. However, single *grlA* mutations are commonly found and this has been taken as confirmation of the hypothesis of topoisomerase IV as the primary quinolone target that mutates first in *S. aureus*. However, in the study of Schmitz *et al* (1998) only two out of 116 strains had *grlA* mutations alone, showing them to be as rare as single mutations in *gyrA*. Although single *grlA* mutations appear to be more frequently isolated than *gyrA* mutations in clinical Gram-positive isolates, this may be because single *gyrA* mutations are very unstable *in vivo*, and therefore are rarely detected. This hypothesis has yet to be investigated. At present the evidence from clinical studies appears to be in favour of *in vitro* findings regarding topoisomerase IV as the primary target; however the number of available studies remains few and there are great risks in drawing firm conclusions from limited data. Although one retrospective resistance progression study in clinical *E. coli* has been described (Gill *et al.* 1999), tracing the development of resistance from quinolone-sensitive to quinolone-resistant in a series of clinical isolates is rarely possible. Employing an accurate animal model of Gram-positive infection that utilises clinically relevant pathogenic strains and therapeutic regimens may be a possible way to investigate the relationship between *in vitro* and *in vivo* data further.

How the role of efflux mutations fits into the process of the development of quinolone resistance is also not well understood. Ferrero *et al* (1995) suggested that efflux mutations, as well as *gyrA* mutations, are secondary events occurring after mutations in *grlA*, based on an *in vitro* model of resistance development. However, an *in vivo* study of the development of low-level quinolone resistance in *S. aureus* has indicated altered efflux may in fact be a primary mutational event, and an alternative to the development of topoisomerase IV mutations (Sulavik and Barg, 1998). In this study low-level quinolone resistance was observed in five first-step *S. aureus* mutants. Four of these strains (ED170, ED209, ED216 and ED229) had no mutations within the QRDRs of *gyrA* and *grlA*. In order to account for the increase in MIC in these strains, overexpression of an efflux pump and *gyrB* mutations were investigated as possible mechanisms of resistance. Sensitivity testing was carried out with tetracycline and ethidium bromide, both of which are efflux pump substrates. Only ED223 showed an elevated MIC of ethidium bromide, while all other strains had identical MICs to the parent strain NCTC 8325/4. Enhanced expression of an efflux system may be observed if the plant alkaloid reserpine is incorporated into a standard sensitivity test (Brenwald *et al.* 1997). Reserpine competitively inhibits efflux of the quinolone rendering the organism sensitive to quinolone concentrations lower than the MIC – effectively the MIC is reduced. However, in this study only strain ED209 showed a lowered MIC in the presence of 20mg/L of reserpine. This suggests that these strains may not be resistant to moxifloxacin by virtue of an enhanced efflux system. Enhanced efflux was investigated more fully by assaying both quinolone accumulation and quinolone efflux from the bacterial cells. In the

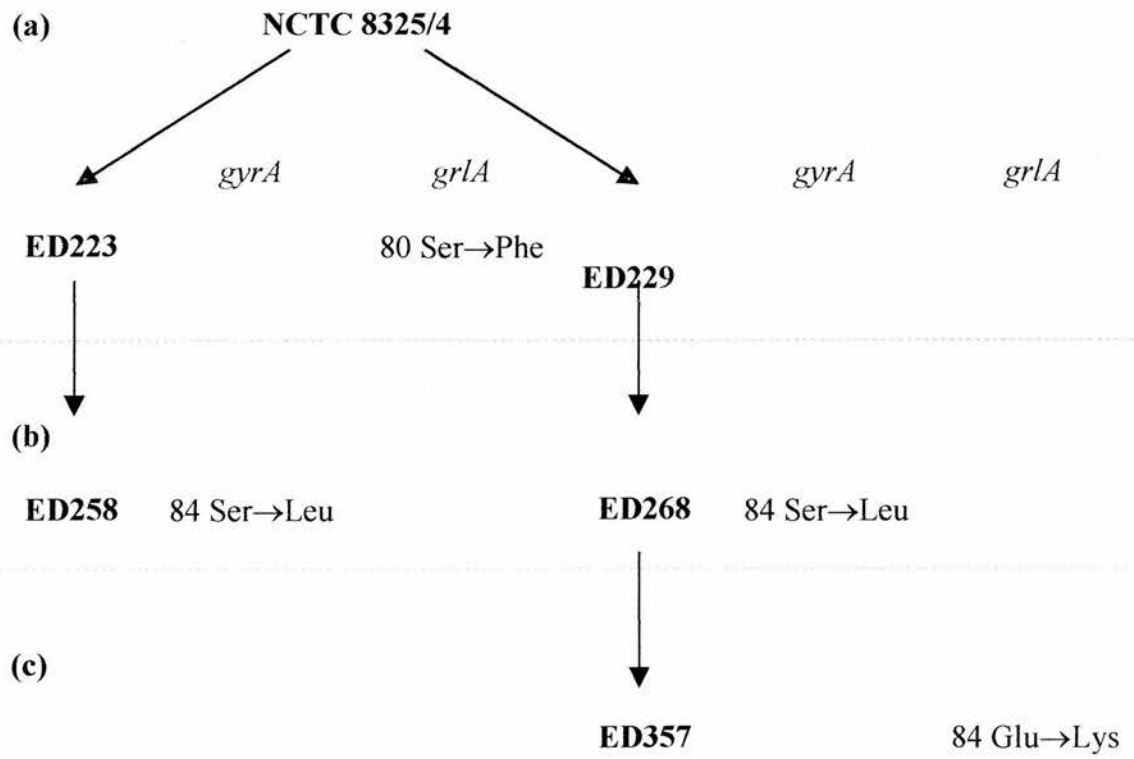
first instance the accumulation of radio-labelled moxifloxacin was not found to be influenced by subsequent addition of CCCP. This agent is a proton motive force inhibitor that should prevent efflux by the NorA protein, leading to greater intracellular accumulation of quinolone (Kaatz *et al.* 1993). In a wild-type strain with normal low-level expression of *norA* efflux pumps (and hence high intracellular quinolone concentrations), the change afforded by the addition of CCCP may be slight. However, the difference between intracellular quinolone concentrations pre- and post-inhibition should be greater if a strain is over-expressing an active efflux pump. In these experiments the addition of CCCP did not have any appreciable effect on the rate of intracellular quinolone accumulation in any of the strains. No difference could be distinguished between the wild-type strain NCTC 8325/4 and the first-step mutants. A washing assay was then employed as a more direct measurement of efflux instead of accumulation. The rate of efflux of radio-labelled moxifloxacin was found to be the same for all strains, and no significant difference in rate was observed when each mutant strain was compared to the parent strain NCTC 8325/4, which has wild-type efflux pump expression. These efflux results confirm those previously obtained by accumulation assays and MICs with reserpine. Although norfloxacin has often been used to study efflux of quinolones, the new generation quinolones are poor efflux pump substrates (MunozBellido *et al.* 1999). Other studies have also shown moxifloxacin to be a poor substrate, largely unaffected by the NorA efflux pump (Piddock and Jin, 1999a; Piddock *et al.* 1999b). However, it was valid to investigate efflux with moxifloxacin instead of norfloxacin

in order to explain the small increases in moxifloxacin resistance in first-step mutants.

From these experiments there was no evidence to suggest that the low-level resistance observed in the first-step mutant strains is due to enhanced efflux by NorA. Another mechanism must therefore have been responsible for resistance in these first-step strains, and mutations in *gyrB* or *grlB* were thought to be relevant. DNA sequencing data revealed that all first-step mutants had identical *gyrB* sequences to the parent strain NCTC 8325/4, excluding mutations in this region from contributing to low level resistance in these strains. However, only a small fragment of the gene was investigated so it is possible that mutations outwith the 327 base region investigated may be present in these strains. Although *grlB* mutations have often been looked for in quinolone resistant clinical isolates of *S. aureus* (Takahashi *et al.* 1998), they have only rarely been found. Such mutations are thought to have a role in the progression to resistance (Tanaka *et al.* 1998), but their precise contribution is unclear as they have always been found in combination with mutations in *gyrA* or *grlA*. The novel nature of these mutations suggests that they may be of lesser importance to the bacterium in terms of enhancing resistance. The results of this thesis suggest that DNA gyrase should be considered as the primary target of moxifloxacin. If this is the case, then mutations within *gyrB* are likely to arise before mutations in *grlB*; however no mutations in *gyrB* were detected. Therefore *grlB* mutations were considered unlikely to be present and were not investigated. Although efflux mutations were not confirmed as a mechanism of

resistance in any of the strains in this study, the pattern of mutation development seems to correlate with the findings of Sulavik and Barg (1998). Figure 8.2 gives a diagrammatic representation of the development of resistance from sensitive to highly resistant strains when selected with moxifloxacin. In Figure 8.2(a) it can be seen that the primary mutation may either be in *grlA* of topoisomerase IV (ED223), or in some as yet undetermined locus, possibly leading to altered efflux (ED229). Both pathways confer the same level of resistance to moxifloxacin. Subsequently, second-step mutations occur in *gyrA* in both pathways (Figure 8.2(b)). Thereafter, a third-step mutation occurs in *grlA* (Figure 8.2(c)). It seems likely that the third mutation following the left fork of the diagram would be an additional *grlA* mutation.

Figure 8.2: Mutation series from strain NCTC 8325/4



The data in this thesis point to DNA gyrase as the primary target for both moxifloxacin and ciprofloxacin in mutants of strain NCTC 8325/4, however mutation studies can only point to targets not prove them. Therefore, in order to confirm the targets of moxifloxacin it will be essential to investigate the effects of individual mutations by site-directed mutagenesis. At least one study has already shown this to be a useful method for evaluating the interaction between the asp87 residue of *gyrA* and quinolones in *E. coli* (Yonezawa *et al.* 1995). Enzyme assays to establish catenation or decatenation, supercoiling, and binding activities of both wild type and mutant DNA gyrase and topoisomerase IV proteins in the presence of quinolones have also been implemented (Gootz *et al.* 1999; Schedletzky *et al.* 1999; Maxwell and Critchlow, 1998; Tanaka *et al.* 1997; Critchlow and Maxwell, 1996; Tanaka *et al.* 1995; Willmott and Maxwell, 1993). However, it is not clear how well inhibitory concentrations of quinolone determined by enzyme inhibition assays correlate with those obtained through standard MIC testing, as little enzyme inhibition is required to inhibit cell growth, while 90% inactivation is required to prevent enzyme activity (Schedletzky *et al.* 1999). Also, since these studies are based on extracellular assays of purified enzymes, it is not clear if the results can be directly related to the clinical situation.

Since the clinical progression of a strain from a sensitive to a resistant phenotype may be completely different to the *in vitro* progression, the effect of therapeutic doses of moxifloxacin on *S. aureus* strains in subcutaneous abscesses in mice was determined. This simple model of infection is perhaps more relevant to human

infection than peritonitis, mastitis or renal abscess models, since *S. aureus* is the leading cause of nosocomial wound infections and is less commonly implicated in these other infections (Bunce *et al.* 1992). Some quinolones, for example ofloxacin and sparfloxacin, have been shown to have a concentration-independent killing effect *in vivo* (Dalhoff, 1999). In the first instance it was established that moxifloxacin was able to kill *S. aureus* in a dose-dependent manner. This result might seem predictable from *in vitro* results but these results often do not correlate with the *in vivo* situation. It was not possible to distinguish between the killing effects of moxifloxacin, trovafloxacin and grepafloxacin when given at concentrations of 0.12mg/L. In an attempt to enhance undetected differences the therapeutic dose was increased to 0.25mg/L, however this did not introduce significant variation in the killing effects of the different agents. Since there was also no statistically significant difference between cohorts of animals treated with antibiotic and the untreated controls at either dose, it would appear that there was an error in the doses given. Each cohort of mice in the dose-response experiment was administered the correct concentration of quinolone, relative to the other cohorts, because the initial stock solution was diluted 1 in 2 each time rather than fresh stocks made up for each individual concentration. Therefore, even if errors were introduced in the actual intra-abscess concentration, dosing group 4 (4 times MIC) had twice the dose of group 3 (2 times MIC), which had twice the dose of group 2 (MIC dose), so any error in the initial stock concentration might not be detected. However, in the quinolone comparison studies where the same single concentration was used for all quinolones given to all mice this kind of problem would show up more readily. In a future study

it would be useful to evaluate the quinolone concentration in abscesses as well as viable counts in order to establish that the appropriate intra-abscess concentration has been achieved.

Interestingly, it was relatively difficult to recover quinolone-resistant mutants from abscess material after therapy when this model was used to investigate the *in vivo* development of resistance. Although the mutation frequencies for selection of first-step *in vivo* mutants were higher than *in vitro* mutants (10^{-5} compared to 10^{-7}) the number of recoverable mutants from treated abscesses was very low. As with the majority of first-step *in vitro* mutants, DNA sequencing of the QRDRs of *grlA* and *gyrA* from first-step *in vivo* mutants did not reveal any mutations to account for the increase in MIC from 0.05 to 0.13mg/L. No second-step mutants could be recovered from first-step *in vivo* strain ED427. However, when second-step selection was repeated with double the inoculum of strain ED170 a single mutant strain was detected with a *gyrA* serine 84 to leucine mutation. From these data, the progression of resistance *in vivo* from sensitive strain to second-step mutant mirrors the *in vitro* situation with progression following the right hand pathway shown in Figure 8.2. However, since the numbers of mutants identified in these experiments were very low, care must be taken in making such an assumption. Also, this would imply that single *gyrA* mutants might be commonly found in the clinical situation, while most studies of clinical isolates indicate that such mutants are rarely recovered (Schmitz *et al.* 1998).

While this *in vivo* study may reflect some aspects of the clinical progression of resistance in *S. aureus* isolates exposed to quinolones, strain NCTC 8325/4 is obviously not a clinical strain. The relatedness of this strain to clinically relevant pathogens has not been investigated, therefore it is not clear how accurately this model may reflect the step-wise development of resistance in clinical isolates. The site of infection, tissue penetration, protein binding, and route of antibiotic administration are some of the factors that influence the outcome of therapy and are thus carefully investigated during the development of a new agent such as moxifloxacin (Stass and Kubitza, 1999; Dalhoff, 1999; Sullivan *et al.* 1999; Siefert *et al.* 1999a; Siefert *et al.* 1999b; Stass *et al.* 1998). These factors affect the *in vivo* concentration of an antibiotic at the site of infection and will therefore also influence the development of resistance, since sub-MIC concentrations are more likely to promote the development of resistance *in vivo* (Doss *et al.* 1995). It would therefore be interesting to investigate the step-wise development of resistance in other models such as the mouse tuberculosis model (Miyazaki *et al.* 1999), the neutropoenic thigh model (Dalhoff, 1999), the prosthetic implant model (Cagni *et al.* 1995) or the endocarditis model (Entenza *et al.* 1999), in order to evaluate whether the results described in this thesis are specific to the subcutaneous abscess model.

Conclusions

This thesis has investigated the mechanisms of action, the step-wise development of resistance and the primary molecular targets of moxifloxacin in *S. aureus*, both *in vitro* and *in vivo*. Moxifloxacin was found to have bactericidal activity against both sensitive and resistant clinical and laboratory-derived strains of *S. aureus*. Time-kill kinetics indicated killing by more than one bactericidal mechanism. Step-wise selection of quinolone-resistant mutants generated strains with mutations in the QRDRs of both *gyrA* and *grlA*. However, the progression of resistance was different from previously published work. The majority of first-step strains had an unknown mutation rather than a change in *grlA* – a single strain had a *grlA* mutation. Subsequent second-step strains all had *gyrA* mutations irrespective of whether the first-step parent had a *grlA* mutation or not. Third-step mutants had high level resistance with one *gyrA*, one *grlA* and one unknown mutation. Similar results were found with both ciprofloxacin-selected mutants and *in vivo* moxifloxacin-selected mutants. These results indicate that DNA gyrase is the primary molecular target in *S. aureus*, and not topoisomerase IV as has been assumed from previous studies. It must be conceded, however, that this conclusion may only be true for strain NCTC 8325/4 and resistant derivatives, as no other strains have been investigated. This study therefore highlights the caution required in interpreting such results, as any general conclusion about molecular targets based on this study may not be applicable to all strains and all quinolones. A study was made of the *in vivo* development of

resistance to moxifloxacin in strain NCTC 8325/4 in a murine subcutaneous abscess model. Topoisomerase IV mutations were rarely selected in first-step mutants, and *gyrA* mutations were selected without *grlA* mutations in second-step mutants. This mutation pattern correlated with previous *in vitro* results pointing to DNA gyrase as the primary mutational target *in vivo*. However, irrespective of whether DNA gyrase or topoisomerase IV is the primary target in *S. aureus*, this study was undertaken with a standard laboratory strain, and it therefore remains unclear how well these results relate to the development of quinolone resistance in clinically significant strains.

Appendix I: Sensitivities of First-step Mutants to Moxifloxacin

Isolate Number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)
NCTC 8325/4*	-	0.05	-
ED170	0.125	0.125	2.5
ED171	0.125	0.150	3
ED172	0.125	0.125	2.5
ED173	0.125	0.125	2.5
ED174	0.125	0.175	3.5
ED175	0.125	0.125	2.5
ED176	0.125	0.125	2.5
ED177	0.125	0.150	3
ED178	0.125	0.150	3
ED179	0.125	0.125	2.5
ED180	0.125	0.175	3.5
ED181	0.125	0.125	2.5
ED182	0.125	0.125	2.5
ED183	0.125	0.125	2.5
ED184	0.125	0.125	2.5
ED185	0.125	0.125	2.5
ED186	0.125	0.125	2.5
ED187	0.125	0.125	2.5
ED188	0.125	0.125	2.5
ED189	0.125	0.150	3
ED190	0.125	0.125	2.5
ED191	0.125	0.150	3
ED192	0.125	0.125	2.5

Isolate Number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)
ED193	0.125	0.100	2
ED194	0.125	0.125	2.5
ED195	0.125	0.100	2
ED196	0.125	0.125	2.5
ED197	0.125	0.100	2
ED198	0.125	0.125	2.5
ED199	0.125	0.100	2
ED200	0.125	0.100	2
ED201	0.125	0.050	1
ED202	0.150	0.150	3
ED203	0.150	0.100	2
ED204	0.150	0.100	2
ED205	0.150	0.100	2
ED206	0.150	0.150	3
ED207	0.150	0.100	2
ED208	0.150	0.075	1.5
ED209	0.150	0.125	2.5
ED210	0.150	0.050	1
ED211	0.150	0.050	1
ED212	0.150	0.050	1
ED213	0.175	0.150	3
ED214	0.175	0.100	2
ED215	0.175	0.075	1.5
ED216	0.175	0.125	2.5
ED217	0.175	0.050	1
ED219	0.175	0.125	2.5
ED220	0.175	0.250	5
ED221	0.200	0.100	2

Isolate Number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)
ED222	0.200	0.125	2.5
ED223	0.200	0.125	2.5
ED224	0.200	0.100	2
ED225	0.200	0.100	2
ED226	0.200	0.100	2
ED227	0.200	0.100	2
ED228	0.200	0.150	3
ED229	0.200	0.125	2.5
ED230	0.200	0.100	2
ED231	0.200	0.125	2.5
ED232	0.200	0.100	2
ED233	0.200	0.125	2.5
ED234	0.200	0.100	2
ED235	0.200	0.125	2.5
ED236	0.200	0.100	2
ED237	0.200	0.125	2.5
ED238	0.200	0.100	2
ED239	0.200	0.125	2.5
ED240	0.200	0.125	2.5
ED241	0.200	0.100	2
ED242	0.200	0.125	2.5
ED243	0.200	0.100	2
ED244	0.200	0.100	2
ED245	0.200	0.100	2
ED246	0.200	0.100	2
ED247	0.200	0.125	2.5
ED248	0.200	0.125	2.5
ED249	0.200	0.125	2.5

Isolate Number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)						
ED250	0.200	0.125				2.5			
ED251	0.200	0.050	1						
ED252	0.200	0.100			2				
ED253	0.200	0.050	1						
ED254	0.200	0.050	1						
ED255	0.200	0.125				2.5			
ED256	0.200	0.100			2				
		Totals	8	2	27	37	9	2	1

* Parent strain

Appendix II: Sensitivities of Second-step Mutants to Moxifloxacin

Parent Strain	Isolate number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)
ED223	ED258	0.4	0.8	16
	ED259	0.4	1.0	20
	ED260	0.4	2.0	40
	ED261	0.4	0.8	16
	ED262	0.6	2.0	40
	ED263	0.8	2.0	40
	ED264	0.8	2.0	40
	ED265	0.8	2.0	40
ED229	ED266	0.4	0.8	16
	ED267	0.4	0.8	16
	ED268	0.4	0.8	16
	ED269	0.4	0.8	16
	ED270	0.4	0.4	8
	ED271	0.4	0.4	8
	ED272	0.4	0.2	4
	ED274	0.4	0.2	4
	ED276	0.6	0.8	16
	ED277	0.8	0.8	16
ED231	ED278	0.4	0.4	8
	ED279	0.4	0.2	4
	ED280	0.4	0.2	4
	ED281	0.4	0.2	4

Parent Strain	Isolate number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)				
	ED282	0.4	0.4		8			
	ED283	0.6	0.8			16		
ED233	ED284	0.4	0.4		8			
	ED285	0.4	0.4		8			
ED235	ED292	0.4	0.8			16		
	ED293	0.4	0.8			16		
	ED294	0.4	0.8			16		
	ED295	0.4	0.4		8			
	ED296	0.4	0.4		8			
	ED297	0.4	0.4		8			
	ED298	0.4	0.4		8			
	ED299	0.4	0.4		8			
ED237	ED300	0.4	0.4		8			
ED239	ED306	0.4	0.8			16		
	ED308	0.4	0.8			16		
	ED309	0.4	0.8			16		
	ED310	0.4	0.8			16		
	ED311	0.4	0.8			16		
ED240	ED312	0.4	0.8			16		
	ED313	0.4	2.0					40
	ED314	0.4	0.8			16		
	ED315	0.4	0.8			16		
	ED316	0.6	0.2	4				
	ED318	0.6	0.8			16		
	ED319	0.6	0.8			16		
	ED320	0.6	1.0				20	
	ED321	0.8	1.0				20	
			Total	6	12	22	3	6

Appendix III: Sensitivities of Third-step Mutants to Moxifloxacin

Parent Strain	Isolate number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)
ED261	ED322	1.8	1.75	35
	ED323	1.8	1.75	35
	ED324	1.8	1.75	35
	ED325	1.8	1.75	35
	ED326	1.8	1.75	35
	ED327	1.8	1.00	20
	ED328	1.8	1.00	20
ED266	ED330	1.2	1.75	35
	ED331	1.2	4.00	80
	ED332	1.2	4.25	85
	ED333	1.2	1.00	20
	ED334	1.2	1.00	20
	ED335	1.2	1.25	25

Parent Strain	Isolate number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)
	ED336	1.8	3.50	70
	ED337	1.8	4.00	80
	ED338	1.8	3.25	65
	ED339	1.8	1.75	35
	ED340	2.4	4.50	90
	ED341	2.4	4.50	90
ED267	ED343	1.8	2.75	55
	ED344	1.8	2.75	55
	ED345	1.8	1.25	25
	ED346	1.8	3.25	65
	ED347	2.4	4.50	90
	ED348	2.4	4.50	90
	ED349	2.4	4.50	90
	ED350	2.4	4.00	80
	ED351	2.4	4.50	90
	ED352	3.0	3.75	75
	ED353	3.0	4.00	80

Parent Strain	Isolate number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)									
	ED354	3.6	2.50				50						
	ED355	3.6	4.00							80			
	ED356	4.2	4.50										90
ED268	ED357	1.2	4.25								85		
	ED358	1.2	4.25								85		
	ED359	1.2	4.00							80			
	ED360	1.2	4.50										90
	ED361	1.2	3.50						70				
	ED362	1.2	2.50				50						
	ED363	1.2	1.50		30								
	ED364	1.8	1.50		30								
	ED365	1.8	4.50										90
	ED366	1.8	4.50										90
	ED367	1.8	4.25								85		
	ED368	1.8	1.50		30								
	ED369	1.8	2.50				50						
	ED370	1.8	2.25			45							

Parent Strain	Isolate number	Selection at (mg/L)	MIC (mg/L)	Change in Resistance (factor x MIC)												
	ED371	1.8	4.50													90
	ED372	1.8	1.75				35									
	ED373	2.4	3.75										75			
	ED374	3.0	4.25												85	
	ED375	3.6	4.25												85	
	ED376	3.6	4.25												85	
	ED377	3.6	4.50													90
	ED378	3.6	2.75							55						
	ED379	3.6	4.25												85	
	ED380	3.6	4.25												85	
	ED381	3.6	4.25												85	
	ED382	3.6	4.25												85	
	ED383	3.6	3.50									70				
	ED384	3.6	4.25												85	
	ED385	4.2	4.50													90
			Total	4	2	3	8	1	3	3	2	3	2	6	12	13

Appendix IV: Statistical Analyses of *In Vivo* Dose Response Data

Table I shows the raw data of triplicate plate counts of cfus recovered from abscess material from animals given 0, 1, 2 or 4 times MIC of moxifloxacin. The means used for statistical analyses are given in Table II. Statistical analyses were performed by Mr Bill Adams, Medical Statistics, University of Edinburgh.

Table I: Raw data – Cfus recovered from staphylococcal abscesses

	Cell counts in triplicate from dilutions of								
Group		**10^{-2}**			**10^{-4}**			**10^{-6}**	
1	-	-	-	19	14	43	0	0	0
1	-	-	-	476	444	308	0	0	3
1	-	-	-	744	816	700	15	8	16
1	-	-	-	65	104	68	8	4	8
1	-	-	-	984	616	1664	37	84	92
1	-	-	-	-	-	-	169	153	115
1	-	-	-	1008	796	832	6	18	12
1	-	-	-	39	36	38	2	1	6
1	-	-	-	356	360	353	4	6	0
1	-	-	-	776	744	-	79	159	84
2	1108	2148	1680	2	1	1	0	0	0
2	2212	1080	-	21	6	7	0	0	0
2	717	690	912	31	1	2	1	0	0
2	2496	-	-	14	44	30	0	0	0
2	840	1136	1970	12	6	0	0	0	0
2	-	-	-	10	23	17	0	1	0
2	-	-	-	45	30	18	0	0	0
2	-	-	-	161	43	59	1	3	0
2	840	1348	268	3	3	3	0	0	1

Group	Cell counts in triplicate from dilutions of								
	10^{-2}			10^{-4}			10^{-6}		
3	1500	711	878	20	33	25	0	0	1
3	-	-	-	53	52	46	1	0	0
3	325	216	344	0	0	0	0	0	0
3	704	208	556	9	28	8	0	1	0
3	524	452	304	7	8	7	0	1	0
3	-	-	-	71	8	7	0	1	0
3	1616	872	1764	23	30	36	0	1	0
3	209	167	166	1	2	2	0	0	0
3	291	384	629	3	1	1	0	0	0
4	96	62	103	0	1	0	0	0	0
4	38	716	180	1	1	0	0	0	0
4	751	1352	680	4	6	5	0	0	0
4	-	716	434	104	94	32	31	24	17
4	374	157	188	2	4	4	0	0	0
4	526	531	632	11	5	5	0	0	0
4	1368	1044	1139	9	9	13	0	0	0
4	2800	-	1556	16	4	7	1	0	0
4	361	816	988	11	8	3	1	0	0
4	993	716	577	-	-	-	-	-	-

Table II: Cfu means used for statistical analyses

Group	Dilution	Cell count	Dilution	Cell Count
1	10^{-4}	25	10^{-6}	0
1	10^{-4}	409	10^{-6}	1
1	10^{-4}	753	10^{-6}	13
1	10^{-4}	79	10^{-6}	7
1	10^{-4}	1088	10^{-6}	71
1	10^{-4}	-	10^{-6}	146
1	10^{-4}	879	10^{-6}	12
1	10^{-4}	38	10^{-6}	3
1	10^{-4}	356	10^{-6}	3
1	10^{-4}	760	10^{-6}	107

Group	Dilution	Cell count	Dilution	Cell Count
2	10^{-2}	1645	10^{-4}	1
2	10^{-2}	1646	10^{-4}	11
2	10^{-2}	773	10^{-4}	11
2	10^{-2}	2496	10^{-4}	29
2	10^{-2}	1315	10^{-4}	6
2	10^{-2}	-	10^{-4}	17
2	10^{-2}	-	10^{-4}	31
2	10^{-2}	-	10^{-4}	88
2	10^{-2}	819	10^{-4}	5
3	10^{-2}	1030	10^{-4}	26
3	10^{-2}	-	10^{-4}	50
3	10^{-2}	295	10^{-4}	0
3	10^{-2}	489	10^{-4}	15
3	10^{-2}	427	10^{-4}	7
3	10^{-2}	-	10^{-4}	62
3	10^{-2}	1417	10^{-4}	30
3	10^{-2}	181	10^{-4}	2
3	10^{-2}	435	10^{-4}	2
4	10^{-2}	87	10^{-4}	0
4	10^{-2}	311	10^{-4}	1
4	10^{-2}	928	10^{-4}	5
4	10^{-2}	575	10^{-4}	77
4	10^{-2}	240	10^{-4}	3
4	10^{-2}	563	10^{-4}	7
4	10^{-2}	1184	10^{-4}	10
4	10^{-2}	2178	10^{-4}	9
4	10^{-2}	742	10^{-4}	7
4	10^{-2}	762	10^{-4}	-

When cell counts are estimated by dilution methods the cell counts are plotted against the dilution factor and data fitted to a straight line through the origin. There is a line for each individual mouse. Unfortunately in this data set the dilutions were too

high or too low leaving only two points, 10^{-4} and either 10^{-2} or 10^{-6}. Thus giving n points on a line

$$\mu_i = k_i c \qquad i = 1 \ldots n \tag{1}$$

or simply $$\frac{\mu_i}{k_i} = c$$

Estimate c, the gradient of the line from μ_i, the number of cell counts and k_i the dilution factor. The sample values of μ_i are r_i the actual cell counts. When working with cell counts the natural distribution to use is the Poisson where the variance of a mean value is equal to the mean. Unfortunately this data has a variation much higher than expected. We get a measure of the variation from the 3 replicates which were made on each reading. Plotting the log values shown in Figure I we get a line with a gradient of 1.50. This means that

$$var(\mu_i) = \mu_i^{1.50}$$

There is also one other source of variation: the mice. Different mice had to be used for different experiments. Thus (1) can be written as

$$\frac{\mu_i}{k_i} = c + \delta m_i \tag{2}$$

where δm_i is the mouse variation.

Figure I: Relationship of the variance to the mean

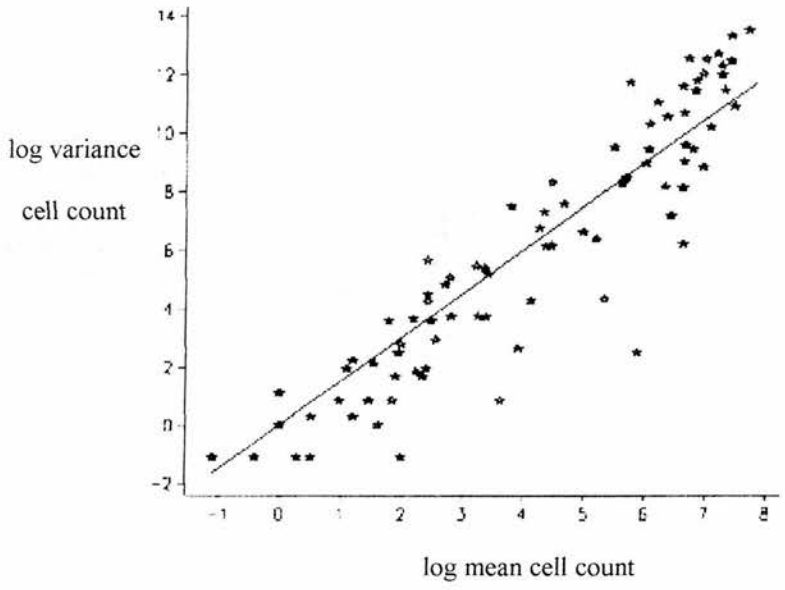


Table III: Mean and sample variance of each group at dilution 10^{-4}

Dosing Group	Raw values		Log values	
	Mean	Variance	Mean	Variance
1	488	158000	5.58	2.049
2	23	710	2.64	1.161
3	23	506	2.41	2.157
4	14	576	1.93	1.408

Looking at Table III it can be seen that for the raw data the variation is ten times higher than expected; the variation is not expected to be the same for each group since the variation is $\propto \mu^{1.50}$. However, the variation between the individual mice should be the same for all 4 groups.

This suggests that equation (2) is replaced by

$$\frac{\mu_i}{k_i} = c \times \delta m_i$$

$$\log \frac{\mu_i}{k_i} = c + \delta m_i \qquad (3)$$

It can be seen in Table III how the log transform has reduced the differences in variation of the 4 groups. All readings need to be standardised for a dilution of 10^{-4}. A straight line is fitted for each mouse, where r_i is the cell count and k_i the dilution.

$$\log r_i = c + k_i \qquad i = 1,2$$

For this problem the method of quasi likelihood and not the ordinary least squares method is used. The method is given by equations (6) and (7).

$$\frac{\partial l_i}{\partial \mu_i} = \frac{r_i - \mu_i}{V_i} \qquad \text{where } V_i = \mu_i^{1.50} \text{ is the variance of } \mu_i$$

$$\frac{\partial l}{\partial c} = \sum_i \frac{r_i - \mu_i}{V_i} \mu \qquad (4)$$

$$-E\left(\frac{\partial^2 l_i}{\partial c^2}\right) = \frac{\mu_i^2}{V_i} \qquad (5)$$

$$= w_i \text{ say}$$

From this the following is obtained

$$c = \frac{\sum w_i \left(\log \frac{\mu_i}{k_i} + \frac{r_i - \mu_i}{\mu_i} \right)}{\sum w_i} \qquad (6)$$

$$\log \mu_i = c + \log k_i \qquad (7)$$

c must be solved in two stages. Into equation (6) put $\mu_i = r_i$ solving the right hand side gives c. Then put c into equation (7); thus a new value for μ_i is determined. This is put back into (6), giving a new value of c. The process is repeated until c remains constant. Estimates of c together with the standard error are given in Table IV.

Table IV: Estimated log values for dilution 10^{-4}

Group 1		Group 2		Group 3		Group 4	
estimate	standard error	estimate	standard error	estimate	standard error	estimate	standard error
3.497	0.398	2.720	0.153	2.470	0.163	-0.082	0.308
5.969	0.214	2.778	0.151	3.938	0.113	1.096	0.229
6.701	0.179	2.099	0.178	1.023	0.233	2.196	0.174
4.959	0.276	3.237	0.134	1.777	0.193	2.509	0.161
7.405	0.150	2.534	0.160	1.536	0.205	0.948	0.238
9.593	0.087	2.872	0.147	4.143	0.107	1.767	0.194
6.822	0.173	3.466	0.127	2.753	0.152	2.468	0.163
4.270	0.328	4.485	0.098	0.639	0.257	3.031	0.141
5.898	0.218	2.075	0.179	1.436	0.211	2.016	0.182
7.425	0.149	-	-	-	-	2.032	0.181

From Table IV the mean and variances for each group are calculated. The standard errors of each estimate obtained from equation (5) using the fitted line are also listed in Table IV. They are all of the same magnitude and are lower than the variances for each group i.e., the variance between mice. Thus the sample variance is used to calculate the standard error. This gives upper and lower confidence limits of each log mean. Anti-logs are taken to return data to the original scale. The results are shown in Table V.

Table V: Estimated mean cell count at 10^{-4} dilution

		Confidence Limits	
Group	Mean	Upper 95%	Lower 95%
1	520.05	1580.1	171.2
2	18.51	30.4	11.3
3	8.94	20.4	3.9
4	6.04	10.8	3.4

Group 4 is significantly lower than group 2. Group 2 has high variance. The variation of the data is unexpectedly high. It should be given by,

$$V_i = \mu_i \qquad \textit{but is instead } \mu_i^{1.5}$$

If more than 2 data points were available for fitting the lines this value could have been attributed to outliers.

Appendix V: Statistical Analyses of Quinolone Comparison at 2 times MIC

When analysing data for cell counts the natural distribution to use is the Poisson. If this is confirmed when the data is tested, it bolsters confidence in the experimental technique. Such a test can be made when 3 replicates are available for each mouse. This is given by

$$x_2^2 = \sum_{i=1}^{i=3} \frac{(r_i - u_i)^2}{u_i} \qquad (1)$$

where r_i is the i'th replicate

u_i is the mean of the 3 readings

These chi square values, with 2 degrees of freedom (d.f.) are given in Table I. There are 2 outliers in this data, values in bold face, 187 and 31. When these are eliminated the probability plot in Figure I is obtained; when the data is Poisson the points should lie about the solid line which is indeed the case.

The next stage is to test for homogeneity in each group for the means in Table I. Denote this mean as

$$m = \frac{\sum u_i}{n}$$

Then if the group is homogenous the standard error (s.e.) of the mean is that of a Poisson

$$s.e.(m) = \sqrt{\frac{m}{n}}$$

The test for homogeneity is

$$x_{n-1}^2 = \sum_i \frac{(u_i - m)^2}{m} \qquad (2)$$

Figure I: Test for Poisson distribution (1) – Probability plot of chi squares

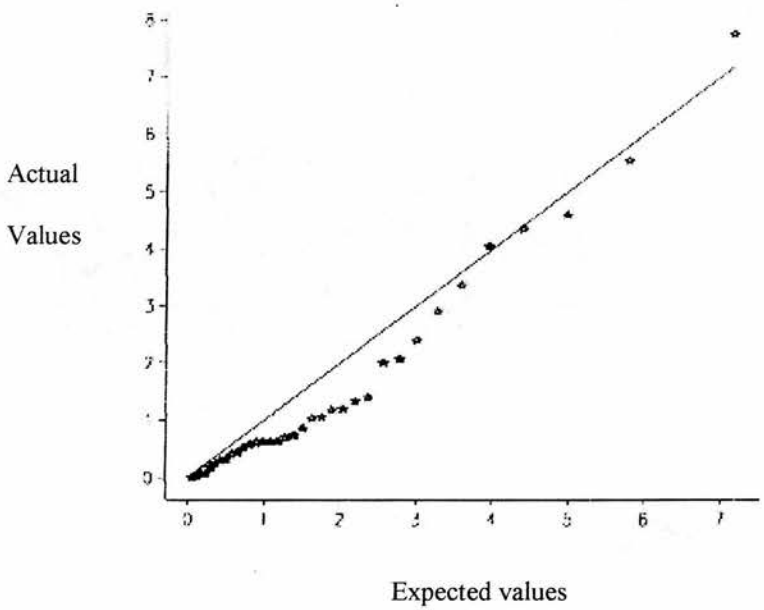


Table I: Data with mean and chi square values

Group	Replicates			Mean u_1	Chi square x_2^2
c	40	38	40	39.33	0.07
c	40	40	38	39.33	0.07
c	53	50	55	52.67	0.24
c	70	**187**	54	103.67	101.72
c	26	28	17	23.67	2.90
c	35	20	17	24.00	7.75
c	18	24	21	21.00	0.86
m	51	37	56	48.00	4.04
m	21	21	18	20.00	0.30
m	53	61	56	56.67	0.58
m	65	60	67	64.00	0.41
m	94	89	103	95.33	1.06
m	20	19	20	19.67	0.03
m	6	8	4	6.00	1.33
m	51	42	32	41.67	4.34
m	27	29	26	27.33	0.17

Group		Replicates		Mean u_1	Chi square x_2^2
t	18	**31**	12	20.33	9.28
t	14	14	14	14.00	0.00
t	29	28	19	25.33	2.39
t	23	33	37	31.00	3.35
t	9	12	6	9.00	2.00
t	164	171	156	163.67	0.69
t	125	117	118	120.00	0.32
t	5	8	6	6.33	0.74
t	5	9	9	7.67	1.39
t	47	72	66	61.67	5.52
g	25	25	30	26.67	0.63
g	108	97	111	105.33	1.03
g	62	87	69	72.67	4.58
g	28	30	34	30.67	0.61
g	65	71	62	66.00	0.64
g	59	53	53	55.00	0.44
g	96	100	111	102.33	1.18
g	15	16	19	16.67	0.52
g	51	47	55	51.00	0.63

The values given in Table II are all highly significant. This means there is a huge natural variation between the mice since from Figure I it cannot be due to sloppy counting. To test for difference in means, the mouse variation (given in Table III) and not that of a Poisson must be used. Box plots were used to get an idea of the distribution of the means in Table I, and these are given in Figures II and III. The horizontal line in each box is the median; the bottom and top are 25% and 75% quantiles. The ends are the maximum and minimum.

Table II: Chi square test for homogeneity (equation 2)

Group	d.f.	x^2
c	6	39.6
m	8	144.4
t	9	586.6
g	8	136.4

Table III: Means and variances

		Raw data		Log values	
Group	**Reps**	**Mean**	**Variance**	**Mean**	**Variance**
c	7	37.43	247	3.546	0.1795
m	9	42.07	759	3.492	0.6889
t	10	45.37	2957	3.210	1.3083
g	9	58.48	997	3.913	0.3919

In Figure II group *t* shows a highly skewed distribution. Transformation to logs in Figure III removes this; the log transformation can pass as normalising the data and t tests can be used to compare means. Unfortunately the variance (Table II) and the ranges (Figure III) show groups *m* and *l* as much higher than *c* and *g*. For unequal variances the equivalent of a t test is the Behrens Fisher test. The biggest difference (*c* from *g*) as a rough test distributed as a t gives

$$t = \frac{3.913 - 3.546}{\sqrt{\frac{6 \times 0.1797 + 8 \times 0.3919}{14} \times \left(\frac{1}{7} + \frac{1}{9}\right)}}$$

$$= 1.32 \qquad \text{a t test with 14 d.f.}$$

which is not significant. In conclusion, it can be seen that there are no significant differences between groups. For the differences to be significant a sample size in each group of about 60 animals, or a more uniform model is required.

Figure I: Distributions – means of 3 replicates shown by box plot of cell counts

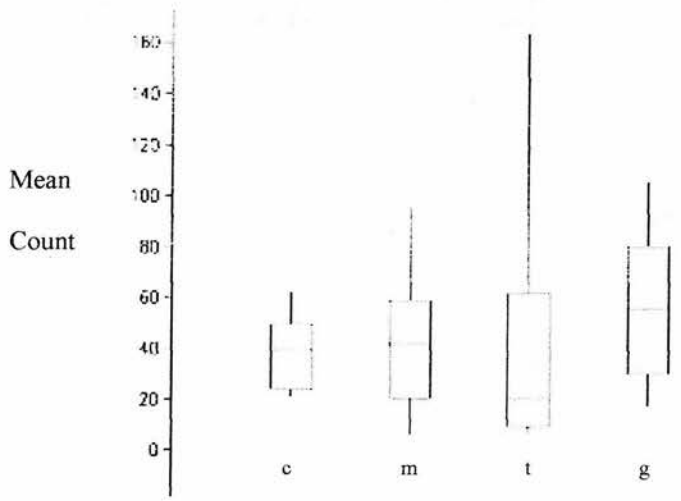
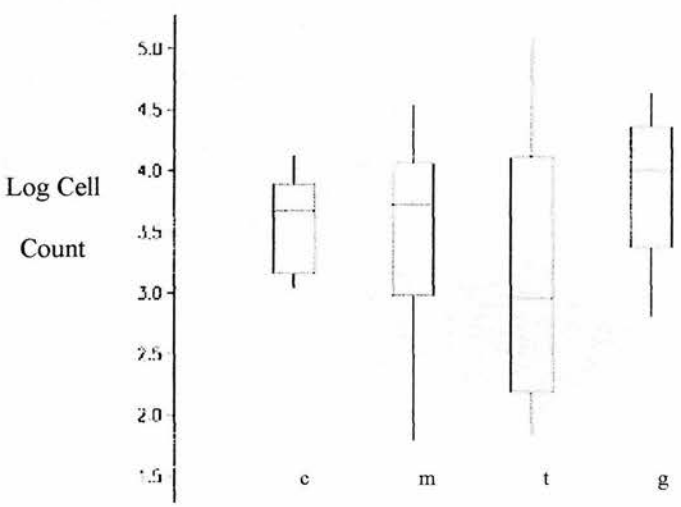


Figure II: Distributions – log transform of mean of 3 replicates



Appendix VI: Statistical Analyses of Quinolone Comparison at 4 times MIC

These statistical analyses were performed with Genstat 5 (release 4.1) by Mr Bill Adams (Medical Statistics, University of Edinburgh). The raw data are given in Table I, and a detailed print out of the program processes follow.

Table I: Raw data of cfus from animals treated with 4 times MIC of moxifloxacin

Group	Triplicate cell counts from 10^{-5} dilution		
c	13	16	18
c	33	51	46
c	52	51	49
c	50	52	57
c	29	38	26
c	27	21	32
m	54	53	51
m	23	12	17
m	19	11	14
m	844	77	26
m	24	15	27
m	110	84	62
m	41	28	11
m	18	25	20
m	78	54	80
m	51	80	76

Group	Triplicate cell counts from 10^{-5} dilution		
t	49	42	51
t	79	99	81
t	14	8	3
t	44	30	25
t	16	9	3
t	11	21	17
t	20	20	22
t	31	48	47
t	49	62	63
g	47	42	47
g	40	43	49
g	9	7	11
g	23	41	29
g	26	22	18
g	11	15	12

1. job 'dur11'
2. open 'data'; ch=2;file=input
3. units [nv=31]
4. factor[lev=4; lab=!t(c, m, t, g) grp; val=!(6(1),10(2),9(3),6(4))
5. read[ch=2] x[1…3]

Table II: Ranges and means

Identifier	Minimum	Mean	Maximum	Values	Missing
x[1]	9.00	37.90	110.00	31	0
x[2]	7.00	37.97	99.00	31	0
x[3]	3.00	35.16	81.00	31	0

6. calc vx=vmean(!p(x[1,2,3]))

7. tabulate [class=grp] vx;means=tt1; var=tt2

8. print tt1,tt2

Table III: Means and variance

Group	Means	Variance
c	36.72	216.1
m	44.17	704.5
t	35.70	661.1
g	27.33	239.0

9. scalar sm[1...4]

10. equate tt1;!p(sm[1...4])

11. for dum1=1...4;dum2=sm[1...4]

12. restrict vs;cond=grp.eq.dum1

13. calc vchi=((vx-dum2)**2)/dum2

14. calc schi=sum(vchi)

15. print schi

16. restrict vx,vchi

17. endfor

schi: *c*=29.42; *m*=173.0; *t*=321.1; *g*=364.8

18. calc lvx=log(vx)

19. delete[red=y] tt1,tt2

20. tabulate[class=grp] lvx;means=ttt1;nobs=ttt2;var=ttt3

21. calc ttt2=ttt3/ttt2

22. calc ttt2=sqrt(ttt2)

23. print ttt1,ttt2,ttt3;dec=3,4,4

Table IV: Log values of means, standard error and variance

Group	Mean (ttt1)	s.e. (ttt2)	variance (ttt3)
c	3.522	0.1900	0.2166
m	3.599	0.2116	0.4478
t	3.310	0.2718	0.6649
g	3.143	0.2713	0.4416

24. treat grp

25. anova[fprob=y] vx

********Analysis of variance********

Variate: vx

Table V: Analysis of variance

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
grp	3	1089.9	363.3	0.71	0.557
Residual	27	13904.2	515.0		
Total	30	14994.1			

MESSAGE: the following units have large residuals.

units 18 50.6 s.e. 21.2

********Tables of means********

Variate: vx

Grand mean 37.0

Table VI: Means

grp	c	m	t	g
	36.7	44.2	35.7	27.3
rep.	6	10	9	6

******Standard errors of difference of means******

Table VII: Standard errors

Table	grp
rep.	unequal
d.f.	27
s.e.d.	13.10 min.rep
	11.72 max-min
	10.15**x** max.rep

(No comparisons in categories where s.e.d. marked with an **x**)

26. anova[fprob=y] lvx

*******Analysis of variance******

Variate: lvx

Table VIII: Variance

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
grp	3	0.9514	0.3171	0.68	0.573
Residual	27	12.6404	0.4682		
Total	30	13.5918			

********Tables of means********

Variate: lvx

Grand mean 3.41

Table IX: Means

grp	c	m	t	g
	3.52	3.60	3.31	3.14
rep.	6	10	9	6

Table X: Standard errors of difference of means

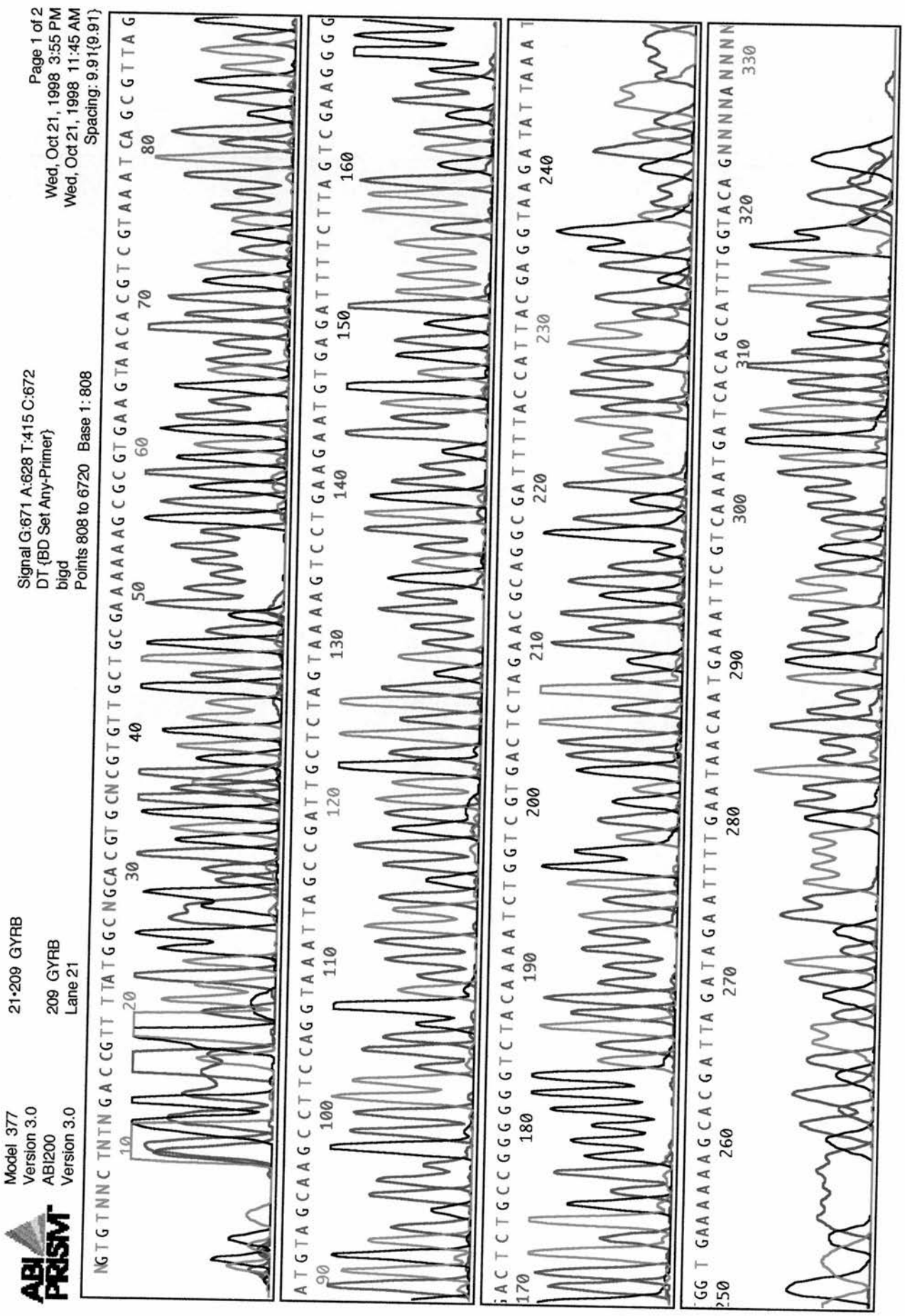
Table	grp
rep.	unequal
d.f.	27
s.e.d.	0.395 min.rep
	0.353 max-min
	0.306**x** max.rep

(No comparisons in categories where s.e.d. marked with an **x**)

27. stop

Conclusion: there are no significant differences between groups.

Appendix VII: Sample Densitometry Trace



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Mechanism of activity of moxifloxacin against *Staphylococcus aureus in vitro*

Esther J Durham,[1] Sebastian GB Amyes,[1] Axel Dalhoff[2] and Christopher J Thomson[3]

[1]*Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK*
[2]*Bayer AG, Pharma Research Centre, Aprather Weg, Postfach 101709, D-42096 Wuppertal, Germany*
[3]*Bayer plc, Pharmaceutical Division, Bayer House, Strawberry Hill, Newbury, Berkshire RG14 1JA, UK*

Abstract

Moxifloxacin was tested against clinical and laboratory strains of *Staphylococcus aureus*. It had good activity against this organism, with MICs of 0.03–0.06 mg/l. Viable counts over a range of moxifloxacin concentrations demonstrated a concentration-dependent bactericidal effect, which was not biphasic as has been seen for a number of earlier quinolones. The optimum bactericidal effect against three strains of *S. aureus* was found to be with 1 mg/l. Two clinical isolates with resistance to methicillin and ciprofloxacin had reduced sensitivity to moxifloxacin. However, moxifloxacin was still able to exert a bactericidal effect against these strains at levels two-fold higher than the MIC. When organisms were tested in the presence of chloramphenicol (a protein synthesis inhibitor) or in phosphate-buffered saline, moxifloxacin still demonstrated a bactericidal effect, albeit reduced. This indicates that moxifloxacin has more than one mode of action against staphylococci.

Introduction

The 1990s have been viewed by some as the 'Armageddon of antimicrobial agents' as more and more multi-drug-resistant pathogens emerge.[1,2] It has become increasingly important to find more effective antimicrobials with which to treat infections caused by such organisms. The quinolone class of antimicrobials targets the interaction of DNA gyrase and topoisomerase IV with DNA, thus inhibiting DNA replication. Although a variety of quinolones has been in clinical use for some time, the mechanisms of quinolone action at the molecular level and how these agents bring about bacterial cell death are still not well understood. Early quinolones had activity predominantly against Gram-negative pathogens and their efficacy against Gram-positive organisms was poor. Multi-drug-resistant Gram-positive organisms, such as *Staphylococcus aureus*, are now recognised as potentially untreatable with traditional antibiotics. In a response to this,

Correspondence to: EJ Durham
Tel: +44 (0) 131 650 8270; email: e.j.durham@ed.ac.uk

new agents with enhanced activity against Gram-positive bacteria are being developed.[3]

Many workers have performed studies to investigate the killing kinetics of quinolones *in vitro*. Smith was the first to demonstrate the now widely accepted biphasic response pattern characteristic of early quinolones.[4] He found that the survival rate of bacteria decreased to a minimum at the optimum bactericidal concentration (OBC) and then began to increase again as the concentration of quinolone increased. This effect was attributed to the inhibition of protein synthesis at concentrations greater than the OBC, a process thought to be required for quinolone action.

Further work[5] showed that when protein synthesis was inhibited by the addition of rifampicin, first-generation quinolones, such as nalidixic acid, were rendered ineffective, but second-generation fluorinated quinolones retained some bactericidal activity. These observations suggested two mechanisms of killing action – mechanism A, which required protein synthesis, and mechanism B, which did not. Since then, a third mechanism, designated C, has also been identified.[6,7] Mechanism C is active against non-dividing cells in phosphate-buffered saline (PBS) but is lost if protein synthesis is inhibited. When a quinolone has more than one mechanism of action, further studies have indicated that the mechanism causing bacterial death may be species specific. For example, ciprofloxacin can kill *Escherichia coli* by mechanisms A and B,[5] but can kill coagulase-negative staphylococci only using mechanism B.[8,9] However, as these studies were performed using standard quinolone-sensitive laboratory strains of bacteria their relevance to clinical pathogens remains unclear.

Moxifloxacin (BAY 12-8039) is a new 8-methoxyfluoroquinolone that has been shown to have good activity against Gram-positive bacteria, including a range of staphylococcal species.[10–12] The aim of this study was to investigate the mechanisms of action of moxifloxacin against both standard laboratory strains and clinical isolates of *S. aureus*.

Methods

Three standard *S. aureus* strains (NCTC 6571, NCTC 8325/4 and E3T) and four clinical isolates were examined. Clinical isolates ED3 and ED5 were resistant to both ciprofloxacin and methicillin, and clinical isolates ED7 and ED9 were sensitive to both ciprofloxacin and methicillin. All clinical isolates were obtained from Edinburgh Royal Infirmary Diagnostic Laboratories. MICs for moxifloxacin and ciprofloxacin against *S. aureus* were determined by a standard agar dilution method (British Society for Antimicrobial Chemotherapy guidelines[13]) on Isosensitest (IST) agar (Oxoid, Basingstoke, UK) using a multipoint inoculator (Denley, Billingshurst, UK) and an inoculum size of 10^6 CFU/ml.

The OBC, defined as the lowest concentration giving the maximum killing effect, was determined for three strains (E3T, NCTC 8325/4 and NCTC 6571) by inoculating nutrient broth (Oxoid) containing a range of moxifloxacin concentrations with log-phase bacteria and incubating for 3 h at 37°C. Viable counts were determined from 0.1 ml samples subcultured on to IST agar and incubated overnight at 37°C.

Killing kinetics of log-phase cultures of clinical isolates ED7 and ED9 (sensitive to ciprofloxacin and methicillin) were determined by exposure of *S. aureus* strains to the OBC for a total period of 210 min. During this period samples were removed every 30 min and viable counts were determined on IST agar plates.

Log-phase cultures of resistant isolates ED3 and ED5 were exposed to 0.5, 1, 2, 4 and 8 × the MIC of moxifloxacin to *S. aureus* (MIC 2 mg/l) in nutrient broth for a total of 210 min. Viable counts were determined from samples removed every 30 min as described above.

Log-phase cultures of NCTC 6571 and NCTC 8325/4 were exposed to the OBC in PBS or in nutrient broth containing a bacteriostatic concentration of chloramphenicol, an inhibitor of bacterial protein synthesis,[14] for 210 min. Viable counts were determined from samples taken every 30 min as described above.

Table 1. *MICs of moxifloxacin and ciprofloxacin against standard and clinical strains of Staphylococcus aureus.*

Strain	Type of strain[a]	MIC (mg/l)	
		Moxifloxacin	Ciprofloxacin
NCTC 6571	Standard	0.03	< 0.06
NCTC 8325/4	Standard	0.06	0.50
E3T	Standard	0.03	< 0.06
ED3 (cip^R, $meth^R$)[b]	Clinical	2.00	> 8.00
ED5 (cip^R, $meth^R$)	Clinical	2.00	> 8.00
ED7	Clinical	0.06	< 0.06
ED9	Clinical	0.06	0.12

[a]Clinical isolate or standard laboratory strain.
[b]Cip^R = ciprofloxacin resistant; $meth^R$ = methicillin resistant.

Results

Table 1 shows the MICs of moxifloxacin and ciprofloxacin against the *S. aureus* strains used. The compounds had similar activity against four of the five methicillin-sensitive strains. One of these strains, NCTC 8325/4 was more susceptible to moxifloxacin than to ciprofloxacin. The MICs of moxifloxacin and ciprofloxacin were increased substantially against the two resistant *S. aureus* strains, although moxifloxacin was still the more active compound.

Figure 1 shows the effect of a range of concentrations of moxifloxacin against three of the sensitive strains. The effect of moxifloxacin is concentration dependent but not biphasic. From these results the OBC would be defined as 1 mg/l.

Figure 2 shows the activity of moxifloxacin at 1 mg/l (OBC) over 210 min against the two sensitive clinical isolates, ED7 and ED9. A good bactericidal effect was seen, with survival decreasing to below 1% by 150 min for both strains.

Figures 3 and 4 show the activity of a range of concentrations of moxifloxacin against the two resistant clinical isolates, ED3 and ED5. The same overall pattern of killing activity was observed for both isolates. Moxifloxacin was not bactericidal at 1 mg/l (the OBC for the sensitive

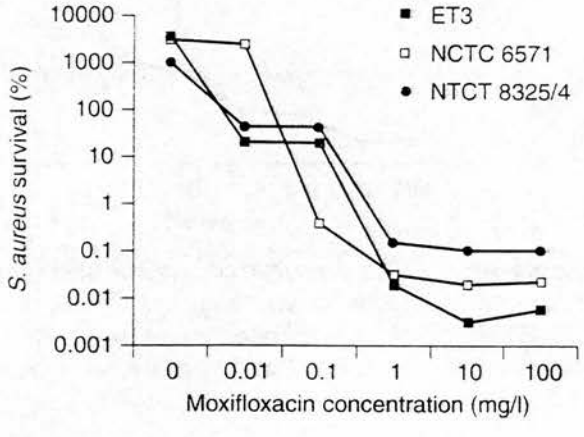

Figure 1. Bactericidal activity of moxifloxacin against *Staphylococcus aureus* strains NCTC 6571, NCTC 8325/4 and E3T.

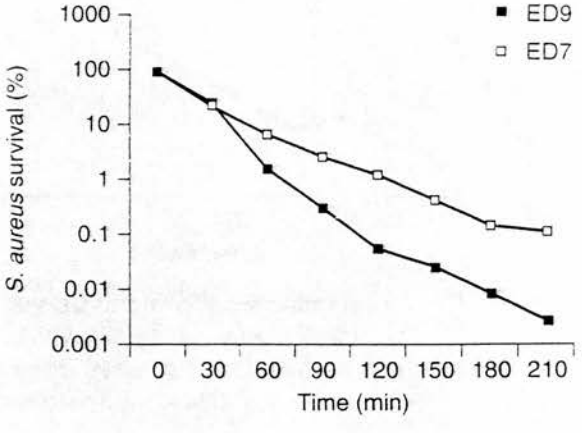

Figure 2. Bactericidal activity of moxifloxacin at 1.0 mg/l against two clinical isolates of *Staphylococcus aureus* in nutrient broth, both sensitive to moxifloxacin (MIC 0.06 mg/l).

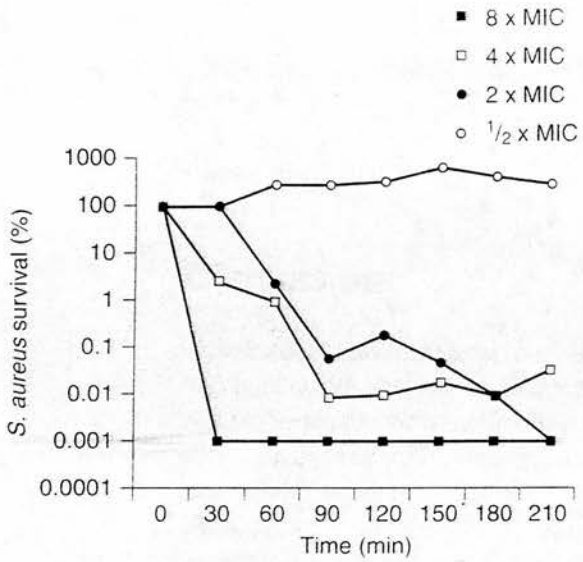

Figure 3. Bactericidal activity of moxifloxacin against ciprofloxacin-resistant *Staphylococcus aureus* isolate ED3 (moxifloxacin MIC 2.0 mg/l).

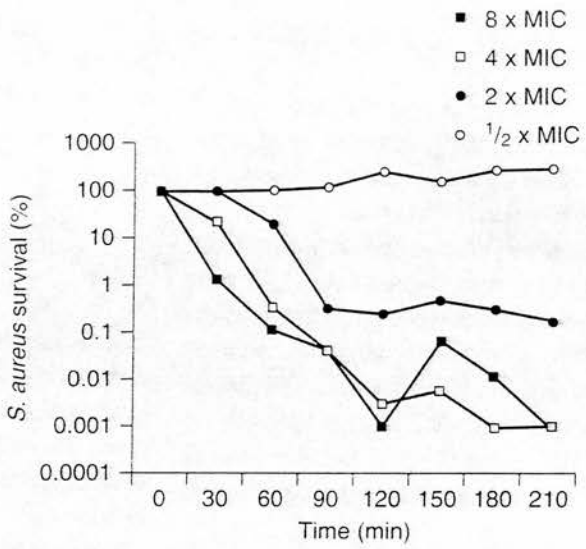

Figure 4. Bactericidal activity of moxifloxacin against ciprofloxacin-resistant *Staphylococcus aureus* isolate ED5 (moxifloxacin MIC 2.0 mg/l).

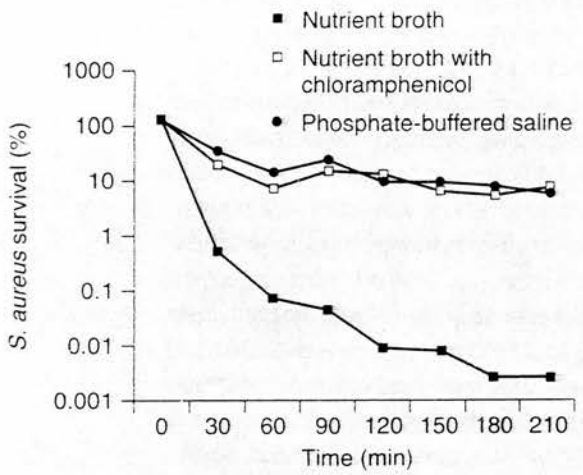

Figure 5. Bactericidal activity of moxifloxacin at 1.0 mg/l against *Staphylococcus aureus* strain NCTC 6571 in the presence and absence of chloramphenicol (moxifloxacin MIC 0.03 mg/l).

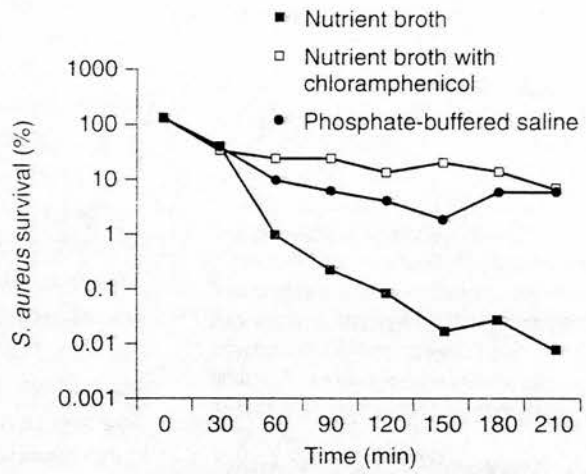

Figure 6. Bactericidal activity of moxifloxacin at 1.0 mg/l against *Staphylococcus aureus* strain NCTC 8325/4 in the presence and absence of chloramphenicol (moxifloxacin MIC 0.06 mg/l).

strains) against these resistant strains. However, activity was restored when the concentration of moxifloxacin was increased, with survival decreasing to less than 1% after only 90 min for both strains at twice the MIC to *S. aureus* (4 mg/l) or higher.

Figures 5 and 6 show the effect of moxifloxacin against two of the sensitive strains, NCTC 6571 and NCTC 8325/4, in the presence and absence of chloramphenicol. Although the efficacy of moxifloxacin was reduced in the presence of chloramphenicol compared with the rate

of kill in nutrient broth alone, it retained some bactericidal activity, killing up to 90% of bacteria. Similarly, the killing activity of moxifloxacin was not completely removed when the organisms were suspended in PBS.

Discussion

Moxifloxacin has been shown by several groups to have good anti-staphylococcal activity[10–12] and to be slightly more active than sparfloxacin[11] and levofloxacin,[10] fluoroquinolones with improved activity against Gram-positive pathogens. Moxifloxacin also compares favourably with other drugs used to treat respiratory tract infections, including cefuroxime, amoxycillin, penicillin G and clarithromycin.[11] In the present study, moxifloxacin was shown to be highly bactericidal against standard laboratory and clinical isolates of *S. aureus* that are sensitive to methicillin and ciprofloxacin. A concentration-dependent effect was seen, but this was not biphasic as has been demonstrated with a number of earlier quinolones.[4] A bactericidal effect was also seen against two methicillin- and ciprofloxacin-resistant clinical isolates. The MIC of moxifloxacin against these isolates was higher than that against sensitive strains, but at concentrations above the MIC a concentration-dependent bactericidal effect was seen. At four times the MIC there was a rapid reduction in bacterial number. Moxifloxacin has also been shown to produce a concentration-dependent bactericidal effect against pneumococci by Klugman and Capper[15] and against *S. aureus* and pneumococci by Dalhoff *et al.*[11]

When organisms were tested in the presence of a protein inhibitor, chloramphenicol, a reduced effect was seen. However, moxifloxacin was still bactericidal, indicating that it has more than one mechanism of action. Similarly, moxifloxacin was still able to kill organisms that were not multiplying (suspended in PBS), lending further support to the idea that this agent has more than one mechanism of action.

Conclusions

These results of this study show that moxifloxacin is bactericidal against both clinical and laboratory strains of *S. aureus* and that while activity is concentration dependent it is not biphasic, as has been observed for older quinolones. Clinical strains of *S. aureus* resistant to ciprofloxacin can still be killed effectively at concentrations of moxifloxacin in excess of the MIC. Moxifloxacin also seems to have more than one mechanism of bactericidal action. Based on the results of these *in vitro* studies, moxifloxacin appears to be a promising bactericidal agent against *S. aureus*.

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Preferred targets of moxifloxacin in *Staphylococcus aureus*

Esther J Durham,[1] Christopher J Thomson,[2] Axel Dalhoff[3] and Sebastian GB Amyes[1]

[1]*Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK*
[2]*Bayer plc, Pharmaceutical Division, Bayer House, Strawberry Hill, Newbury, Berkshire RG14 1JA, UK*
[3]*Bayer AG, Pharma Research Centre, Aprather Weg, Postfach 101709, D-42096 Wuppertal, Germany*

Abstract

A series of mutants of a strain of *Staphylococcus aureus* was obtained by plating the bacteria out on agar containing a range of concentrations of the fluoroquinolone moxifloxacin. Mutants growing at concentrations above the original MIC were selected and re-plated in three consecutive steps. The MICs of moxifloxacin, ciprofloxacin, sparfloxacin and trovafloxacin were determined against five mutants from each stage. Although the MICs of all compounds tested increased sequentially, moxifloxacin and trovafloxacin were the least affected of the compounds. Genomic DNA was extracted from the 15 strains and the polymerase chain reaction (PCR) was used to amplify the quinolone resistance-determining region of *gyrA* and *grlA*. Four of the first-step mutants had no *gyrA* or *grlA* mutations, but one strain had a *grlA* mutation that was not accompanied by an increase in the MIC. Three different *gyrA* mutations were present in the second-step mutants, accompanied by mutations in *grlA* in only two of the strains. All five third-step mutants (those with the highest MICs) had identical mutations – Ser-84 to Leu in *gyrA* and Glu-84 to Lys in *grlA*. These results suggest that topoisomerase may not be the most important target in *S. aureus*.

Introduction

The quinolones are a class of antimicrobials that inhibit DNA synthesis by targeting bacterial DNA gyrase and topoisomerase IV. Although their exact mechanisms of action have yet to be determined, recent evidence suggests that DNA gyrase is the primary quinolone target in Gram-negative organisms while topoisomerase IV is the primary target in Gram-positives.[1–3] However, some studies indicate that this is not the full story, as new quinolones may have different targets from their older counterparts within a single species.[4]

Resistance to quinolones arises through changes within the genes that encode for DNA

Correspondence to: EJ Durham
Tel: +44 131 650 8270; email: e.j.durham@ed.ac.uk

gyrase (*gyrA* and *gyrB*) or topoisomerase IV (*grlA* and *grlB* in *Staphylococcus aureus*). Sequencing of *gyrA* and *grlA* genes from resistant mutants has identified 'hot spots' of mutations, which are commonly referred to as the quinolone resistance-determining regions or QRDRs.[5–9] In Gram-negative organisms, topoisomerase IV mutations contribute to quinolone resistance to a lesser degree than gyrase mutations, and are believed to play a secondary role. In Gram-positive organisms the situation seems to be reversed, and gyrase mutations do not seem to produce high-level quinolone resistance in such bacteria unless certain topoisomerase IV mutations are already present.[3,5,6,10,11] However, in many mutants with altered quinolone susceptibility no relevant changes to the QRDRs of either the gyrase or topoisomerase IV genes have been detected,[12–14] and resistance may be attributed to an efflux system encoded by *norA*.[15] Mutation in the *norA* gene leads to enhanced efflux of quinolones, which has been shown to cause moderate, but clinically relevant, levels of resistance in *S. aureus*.[12–14,16]

Resistance to quinolones in *S. aureus* is unusual in that a number of methicillin-resistant strains, although resistant to older compounds (norfloxacin, enofloxacin, ofloxacin and ciprofloxacin), remain sensitive to some of the newer fluoroquinolones. This has been explained in recent studies showing that classic gyrase and topoisomerase mutations associated with resistance affect the newer quinolones to a lesser degree than the older compounds.[9,17–19] Single-step mutants resistant to older quinolones can be obtained, but these are not usually resistant to the newer compounds. Mutants with more than a one-step change seem to be required for resistance in DNA gyrase.[20] This suggests that new quinolones may interact differently with their targets or have different or additional targets compared with older quinolones.

Moxifloxacin is a new 8-methoxyquinolone with enhanced activity against Gram-positive organisms. It has been shown to be less influenced than older quinolones by known mutations in *gyrA*, *gyrB*, *grlA* and *grlB*.[21] The purpose of this study was to determine the intracellular targets of moxifloxacin in *S. aureus* by sequence analysis of the QRDR of *gyrA*, the gene encoding for DNA gyrase, and the analogous region of *grlA*, encoding for topoisomerase IV.

Methods

First-, second- and third-step quinolone-resistant mutants of *S. aureus* were selected from a standard sensitive parent strain (NCTC 8325/4) on a series of Mueller–Hinton agar plates (Oxoid, Basingstoke, UK) containing concentrations of moxifloxacin at or above the MIC (Figure 1). The MICs of moxifloxacin, ciprofloxacin, trovafloxacin and sparfloxacin (supplied by Bayer AG, Wuppertal, Germany) for the parent strain and all mutants were initially determined by the standard agar dilution method described in the British Society for Antimicrobial Chemotherapy sensitivity testing guidelines.[22] Subsequently, more precise values for moxifloxacin were determined by using a narrow range of concentrations around the value determined as the MIC by standard methods, in order to ascertain the optimum starting concentration for subsequent selection plates.

First-step mutants of the sensitive parent strain were selected from colonies growing on plates containing 0.125 mg/l of moxifloxacin (2.5 × the MIC). Second-step mutants were derived

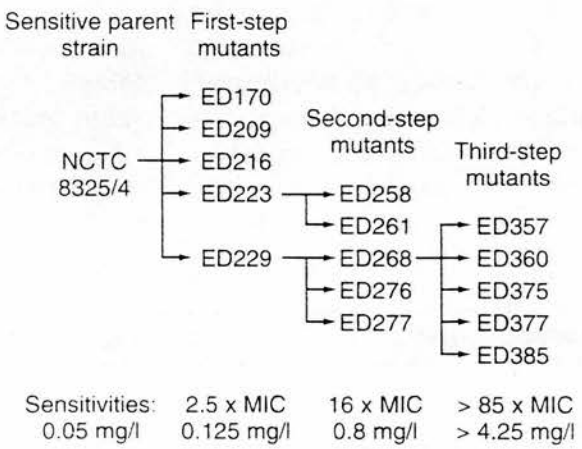

Figure 1. Moxifloxacin-resistant mutants of *Staphylococcus aureus* selected *in vitro*.

from the first-step mutants by plating out these mutants on a range of moxifloxacin concentrations and selecting those colonies growing at ≥ 2.5 times the MIC. Third-step mutants were derived from second-step mutants with MICs of 0.8 mg/l (16 × the MIC of NCTC 8325/4). Viable counts of all parent strains were determined at each mutation step by subculturing on to non-selective plates to determine mutation frequencies. These were calculated by dividing the number of colonies obtained on selective plates by the number of viable organisms per 100 µl when the same stock culture was spread on to non-selective plates.

Five mutants chosen from each mutation step were characterised by DNA sequencing. A rapid extraction method[23] was used to extract genomic DNA from whole staphylococcal cells, and 10 µl of extract were used for each polymerase chain reaction (PCR) to amplify the QRDR of *gyrA* and the analogous region of *grlA*. Oligonucleotide primers (Immunogen International Ltd, Sunderland, UK), detailed in Table 1, were combined with other PCR reagents as follows: 10 pmol of each primer, 200 µM dNTPs, 2.5 mM $MgCl_2$ and 2 units of *Taq* DNA polymerase (all from Promega, Southampton, UK). Thermal cycling was performed with an initial denaturing step of 94°C for 30 s, 55°C for 30 s and 72°C for 10 min. This was followed by 30 cycles of 1 min at 90°C for denaturation, 2 min at 42°C for annealing and 3 min at 72°C for polymerisation. The final extension step took place for 5 min at 72°C.

Purified PCR products were subjected to cycle sequencing using an ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit according to the manufacturer's instructions (Perkin-Elmer, Seer Green, UK). Extension products were purified by rapid ethanol precipitation and sequences determined by automatic sequencing on an ABI PRISM DNA sequencer (Perkin-Elmer). All strains were sequenced twice from different PCR products to confirm the results.

Results

The sensitivities of all the mutant strains isolated are shown in Table 2. Moxifloxacin and trovafloxacin were slightly more active than sparfloxacin, which was more active than ciprofloxacin against all strains. MIC values for all four compounds increased gradually from first-step mutants to third-step mutants.

Table 3 shows the mutation frequencies of the three different types of mutants and the concentration of moxifloxacin at which they were isolated. Overall, the frequencies ranged between 3.3×10^{-7} and 1.7×10^{-12}, the highest values being found for the first-step mutants, and decreasing for subsequent generations of mutants with higher levels of resistance.

Table 4 shows the changes in the QRDRs of the 15 mutants. It can be seen that four first-step mutants with moxifloxacin MICs of 0.125 mg/l (2.5 × the MIC of the parent strain, NCTC 8325/4) had no *gyrA* or *grlA* mutations. Although the MIC of the fifth strain (ED223) was also 0.125 mg/l and it had no *gyrA* mutations, there was a Ser-80 to Phe change in *grlA*. All of the second-step mutants had an MIC 16 times that of the parent strain, although three different mutations (Ser-84 to Ala or Leu, or Glu-88 to Lys) were present in *gyrA*. Only two strains, ED258 and ED261, had a change of Ser-80 to Phe in the *grlA* gene inherited from their parent strain.

Table 1. Primer sequences.

Primer	Sequence	Size of amplified fragment
gyrA 5′	GAC TTC TAA GCG CTG TGA AC	
gyrA 3′	AAG TTA CCT TGG CCA TCA AC	374 bases
grlA 5′	TGT TTT AGG TGA TCG CTT TGG	
grlA 3′	GGC AAT ACC ATT GGT TCG AG	434 bases

Table 2. MICs (mg/l) of four quinolones against 15 mutants of *Staphylococcus aureus*.

Mutation step	Strain	Ciprofloxacin	Sparfloxacin	Trovafloxacin	Moxifloxacin
Parent	NCTC 8325/4	0.5	0.06	0.06	0.06
First step	ED170	2	0.25	0.12	0.12
	ED209	1	0.06	0.06	0.12
	ED216	2	0.06	0.12	0.12
	ED223	2	0.25	0.25	0.12
	ED229	1	0.06	0.12	0.12
Second step	ED258	8	1	1	1
	ED261	8	1	1	1
	ED268	2	1	0.25	1
	ED276	2	1	0.25	1
	ED277	2	1	0.5	1
Third step	ED357	32	16	4	8
	ED360	32	16	4	8
	ED375	32	16	4	8
	ED377	32	16	4	8
	ED385	32	16	4	8

Table 3. Mutation frequencies of 15 moxifloxacin-resistant mutants of *Staphylococcus aureus* NCTC 8325/4.

Mutation step	Parent strain	Moxifloxacin concentration (mg/l)	Multiple of MIC	Mutation frequency
First step	NCTC 8325/4	0.125	2.5	3.3×10^{-7}
		0.150	3	8.6×10^{-8}
		0.175	3.5	6.5×10^{-8}
		0.200	4	3.5×10^{-7}
Second step	ED223	0.4	8	5.9×10^{-11}
	ED229	0.4	8	2.0×10^{-10}
		0.6	12	2.5×10^{-11}
		0.8	16	2.5×10^{-11}
Third step	ED268	1.2	24	1.4×10^{-11}
		3.6	72	1.7×10^{-11}
		4.2	84	1.7×10^{-12}

This did not alter the MIC of moxifloxacin, but the ciprofloxacin MIC increased four-fold. The other three second-step mutants had no changes in *grlA*, only in *gyrA*. All third-step mutants had a Glu-84 to Lys change in *grlA* accompanied by a Ser-84 to Leu change in *gyrA*, and had MICs of 4.25–4.5 mg/l (85–90 × the MIC of the parent strain).

Discussion

In the past, work by several groups has suggested that quinolone antibacterials have preferential targets within bacteria depending on whether the organism is Gram-positive or Gram-negative. Until recently,[21] it had been suggested by many workers that a prerequisite for low-level

Table 4. Changes within the quinolone resistance-determining regions of *gyrA* and *grlA* of 15 moxifloxacin-resistant mutants compared with the parent strain (*Staphylococcus aureus* NCTC 8325/4).

Mutation step	Mutant	MIC[a] (mg/l)	*gyrA* mutation			*grlA* mutation		
			Codon position	Base change	Amino acid change	Codon position	Base change	Amino acid change
Parent	NCTC-8325/4	0.05		None			None	
First step	ED170	0.125		None			None	
	ED209	0.125		None			None	
	ED216	0.125		None			None	
	ED223	0.125		None		80	TCC→TTC	Ser→Phe
	ED229	0.125		None			None	
Second step	ED258	0.8	84	TCA→GCA	Ser→Ala	80	TCC→TTC	Ser→Phe
	ED261	0.8	84	TCA→GCA	Ser→Ala	80	TCC→TTC	Ser→Phe
	ED268	0.8	84	TCA→TTA	Ser→Leu		None	
	ED276	0.8	84	TCA→TTA	Ser→Leu		None	
	ED277	0.8	88	GAA→AAA	Glu→Lys		None	
Third step	ED357	4.25	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
	ED360	4.5	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
	ED375	4.25	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
	ED377	4.5	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys
	ED385	4.5	84	TCA→TTA	Ser→Leu	84	GAA→AAA	Glu→Lys

[a]Narrow-range MIC.

resistance was a mutation within either *gyrA* or *grlA*;[2] however, the results of this study show four first-step mutants with a clear, albeit small, increase in MIC which cannot be attributed to such mutations. This implicates some other resistance mechanism, such as *gyrB* or efflux mutations, both of which have been shown to cause small decreases in sensitivity.[15,24]

Three second-step mutants were found to have *gyrA* mutations in the absence of *grlA* mutations, despite the fact that *S. aureus* is a Gram-positive organism. This casts some doubt on the theory that topoisomerase IV is the primary target for quinolones in Gram-positive organisms. Other workers have suggested that the selective mutation specificity may be related to quinolone structure rather than organism classification[2] or that quinolones may differ in their sensitivity to the particular enzyme.[20] However, as the structure–function relationships of quinolones is still only poorly understood, it is difficult to be conclusive with such arguments.

Although other workers[2,11] have ascribed a key role in the development of high-level resistance to changes within codon 80 of *grlA*, the Ser-80 to Phe change observed in one first-step and two second-step mutants did not confer an increase in resistance to moxifloxacin, even when combined with a mutation in *gyrA*. In contrast, however, the Glu-84 to Lys mutation observed in all third-step mutants had a considerable effect by increasing the MIC by more than five-fold, from 0.8 to 4.5 mg/l. This result agrees with previous evidence that mutations which have an effect on the sensitivity to older quinolones may not have such a marked effect on the sensitivity to newer fluoroquinolones, such as moxifloxacin. This may be because the newer agents have different targets from the older agents.[17–19] The reason why gyrA mutations were found before *grlA* mutations in most of our strains – a result in contrast to those of previous studies – might be a consequence of strain specificity. This seems unlikely, however, as previous studies using a variety of strains produced similar results to each other. A possible explanation lies in the methodology used: most groups used much higher selection concentrations than those used in this study, and this may affect which mutation appears first. If this is the case, it is difficult to identify, at this stage, which might be more important clinically.

Our results suggest that topoisomerase IV is not necessarily the primary target of moxifloxacin in *S. aureus*, and that drug-specific interactions may be as important as the bacterial species in determining the stepwise development of resistance.

Conclusions

A series of *S. aureus* mutants with decreased sensitivity to moxifloxacin was isolated by sequential plating out and changes in the QRDRs of *gyrA* and *grlA* were examined. The increase in MIC found with these mutants was less with moxifloxacin and trovafloxacin than with ciprofloxacin and sparfloxacin. Only one of the first-step mutants had any changes in either *gyrA* or *grlA* to account for the increase in MICs. Three of the second-step mutants, those with a moderate degree of resistance to moxifloxacin and ciprofloxacin, had mutations only in *gyrA*, indicating that gyrase may be more important than topoisomerase in these strains. When changes were seen in both *gyrA* and *grlA*, this was accompanied by a greater increase in resistance, especially to ciprofloxacin. These results indicate that the newer quinolones, such as moxifloxacin, may not show the same reduction of activity as older quinolones against staphylococci.

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